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IN MEMORIAM
THOMAS CLEMENT CHENG
NOVEMBER 5, 1930–NOVEMBER 28, 2000

Thomas C. Cheng, an international authority in the fields of parasitology, molluscan biology, and shellfish diseases died on November 28, 2000 in Charleston, South Carolina. He was 70 years old and is survived by three children, Thomas, Jr., Brad, and Allison.

Tom was born on November 5, 1930 at Nanking, China. He moved to the United States with his parents he was six years old. Tom spoke very little English but arrived without any problems and soon attended local public schools. After graduating from Greenbrier Military School, Lewisburg, West Virginia he attended Wayne State University at Detroit, Michigan, with a major in biology. While at Wayne State, he enrolled in a parasitology course taught by Professor Dominc L. DeGiusti and became very interested in the public health importance of parasites. After graduation, he joined the U.S. Army and for four years he worked in a clinical diagnostic laboratory performing parasitology, serology, and bacteriology during the Korean conflict. These experiences triggered even a greater interest in parasites, and upon discharge from military service, he enrolled in the graduate program at the University of Virginia and received his M.S. in biology in 1956, and his Ph.D. in 1958 under the of codirection of Professor Bruce Dodson Reynolds and Professor Horton Holcome Hobbs, Jr.

His first faculty position was at the University of Maryland, Baltimore, Maryland as an instructor-assistant professor. One year later, he joined the Department of Biology at Lafayette College, Easton, Pennsylvania. During his six years at Lafayette College, he taught various courses including parasitology, microbiology, general biology, physiology, and invertebrate zoology. During these six years, he formulated and wrote his first book, *The Biology of Animal Parasites*. The book became a popular textbook for an introductory course in parasitology. He was awarded a National Institutes of Health Fellowship in 1964 to travel to the Pacific Biomedical Research Center at the University of Hawaii, Honolulu, Hawaii. He worked as a visiting scientist on the modes of infection of *Achatina fulica* by the larvae of *Angiostrongylus cantonensis*. After this one-year fellowship, he returned to the U.S. mainland and was appointed Chief of the Immunology and Parasitology section at the U.S. Public Health Service Northeast Marine Health Sciences Laboratory at Narrangansett, Rhode Island. It was here that he started one of his most significant academic accomplishments, the writing of *Marine Molluscs as Host for Symbioses with a Review of Known Parasites of Commericially Important Species* that was published in the 5th volume of *Advances in Marine Biology*.

He then returned to the University of Hawaii to join the Department of Zoology. After five years, he returned to Pennsylvania and created and directed the Institute for Pathobiology at Lehigh University. The next 12 years were very productive and exciting for Tom. However, 1980, Tom was approached by the Medical University of South Carolina and asked if he would be interested in creating a Marine Biomedical Research Program. He accepted their offer and the challenge and joined the Medical University at South Carolina

at Charleston, South Carolina. He served as Director of this program until his retirement in 1993. While the Director of the Marine Biomedical Research Program, he accepted the position as Acting Chairman of the Department of Cell Biology and Anatomy of the Medical University for 18 months. It was during this time that he was recognized by the scientific community for his significant contributions to the advancement of molluscan immunology and host parasite interactions. He received numerous awards including a Senior Fulbright Scholar, Directeur de Recherche Award, and the Montpellier Medal and was in very active in lecturing and chairing many national and international meetings and congresses.

Tom retired in 1993, but still remained active at the laboratory bench working on molluscan diseases and immunology until the end. During his seven years of retirement, he continued to write research grants and gain funding for his ideas. When diagnosed with terminal esophageal cancer, his pace slowed but not his interest in his research. He continued to work daily for several hours until he could no longer fight the fatigue and pain. Tom Cheng was a bright and energetic teacher and scientist who helped shaped the careers of many individuals who worked in the areas of parasitology and molluscan immunology. He published over 350 scientific papers, 22 books, and served as Editor of the *Journal of Invertebrate Pathology* for twenty-three years and twenty years as coeditor of *Experimental Parasitology*. He directed twenty-three graduate students and ten postdoctoral fellows.

The field of molluscan shellfish pathobiology and parasitology will miss Tom Cheng. However, his contributions to these important areas will continue to live through his scholarly works, his students, and colleagues he touched during his remarkably productive life.

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ANOTHER INTRODUCED MARINE MOLLUSK IN THE GULF OF MEXICO: THE INDO-PACIFIC GREEN MUSSEL, *PERNA VIRIDIS*, IN TAMPA BAY, FLORIDA*

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ABSTRACT In July 1999, green mussels were seen for the first time at Tampa Electric Company's (TECO) Gannon Station Power Plant (Gulf of Mexico, Hillsborough Bay portion of Tampa Bay, western coast of Florida) during routine maintenance of the seawater intake system. The mussels were identified as *Perna viridis* (Linnaeus 1758) based on shell and anatomical characters. This identification has been confirmed by cytological analysis. *Perna viridis* had previously not been found in the United States or in the Gulf of Mexico. *Perna viridis* is a native species in the Indian and Pacific Oceans and was discovered in the Caribbean Sea in Trinidad in 1990 and in Jamaica in 1998. The populations are widespread locally and well established; the largest specimen collected was estimated to be 15 months of age. This is the second species of *Perna* introduced to the Gulf of Mexico, joining *Perna perna* (Linnaeus 1758), which was discovered in Texas in 1990. Since the initial discovery of *Perna viridis* in Tampa Bay, range surveys have found it as far north as John's Pass in St. Petersburg, Pinellas County, Florida and as far south as Boca Grande, Charlotte County, Florida.

KEY WORDS: *Perna viridis*, Bivalvia, exotic species, invading species, Mytilidae, Florida, aquaculture

INTRODUCTION

The introduction of non-native species through natural, intentional or accidental transport by human activities is a threat to natural flora and fauna and can cause severe economical impact (Meineke 1999). According to Carlton (1992), the major human activities resulting in the introduction of non-native species in North America are the following: the transportation of organisms on the outside (fouling species) or on the inside (boring species) of ships; the transportation of organisms inside vessels in solid ballast (rocks, sand and detritus) or ballast water from coastal, transoceanic, and interoceanic vessels; the transport of oysters (including organisms, sediment and detritus on or in the oyster shells); and the intentional release of species for fisheries purposes. United States coastal and inland waters now support a large list of invasive bivalve species [e.g., *Perna perna*, *Mytilus galloprovincialis* Lamarck 1819, *Dreissena polymorpha* (Pallas 1771), *D. bugensis* Andrusov 1897, *Musculista senhousia* (Benson 1842), *Corbicula fluminea* (Müller 1774); see Turgeon et al. (1998) for full list].

In July 1999, Tampa Electric Company (TECO) requested Mote Marine Laboratory (Sarasota, Florida) to identify "green mussels" that were discovered fouling the water intake structures of TECO Gannon Station Power Plant in the Hillsborough Bay portion of Tampa Bay, Florida (27°54′25″N; 81°25′35″W). The green mussels were immediately recognized as members of the tropical/subtropical genus *Perna* comprised of three extant species: *P. perna* (Linnaeus 1758), *P. viridis* (Linnaeus 1758), and *P. canaliculus* (Gmelin 1791) (reviewed by Siddall 1980a). The three *Perna* species are similar morphologically and exhibit considerable variation in taxonomically important morphological characters (Siddall 1980a; Sadacharan, 1982, Holland et al. 1999), making it difficult to separate the species.

In the past, *Perna* species were easily distinguished from one another on the basis of their mutually exclusive geographical distributions. *Perna perna* occurs natively in warm tropical and subtropical coastal regions of South America and Africa (Jeffs et al. 1999) and also extends into the Mediterranean (Berry 1978). *Perna viridis* is a tropical euryhaline species, widely distributed natively throughout the Indo-Pacific having a western limit from the Persian Gulf, an eastern limit at Papua New Guinea (Morton 1987), and northern limit to southern Japan (Cheung 1993), including southern China (Huang et al. 1983). *Perna canaliculus* is only found in coastal waters of New Zealand (Siddall 1980a) and is found on North, South, and Stewart Islands but is most abundant in central and northern New Zealand (Powell 1979; Jeffs et al. 1999).

Today, these species can no longer be distinguished from one another based only on geographical location. Segnini de Bravo et al. (1998) mention that *Perna perna* colonized the Caribbean Sea several decades ago. Diaz Merlano and Hegedus (1994) report a series of fresh shells found on the beach near Bocachia along the Columbian coast. The geological range of *P. perna* expanded again in 1990 when two small specimens (2 cm in length) were collected in the western Gulf of Mexico from the Port Aransas Jetty on the east coast of Texas in February 1990 (Hicks & Tunnell 1993). The Texas *P. perna* are now found from the mouth of the Colorado River, Texas south to Veracruz, Mexico (Sea Grant News Media Center Website 1999). The geographical range of *P. viridis* has also expanded. Morton (1987, p. 159) reports "Siddall (1980a) does not consider *P. viridis* to be naturally distributed along the coast of China or Japan and Arakawa . . . believes the species was introduced into Japan sometime around 1967." In 1990 *P. viridis* was first observed in the southern Caribbean Sea at Port Lisas in Trinidad and by 1992 had spread along the entire Trinidad coast of the Gulf of Paria (Agard et al. 1992). In 1993 *P. viridis* appeared on the Venezuelan coast of the Gulf of Paria and by 1995 was discovered in Isla Margarita (Segnini de Bravo et al. 1998). According to Segnini de Bravo et al. (1998), *P. viridis* is driving *P. perna* from its natural beds in La Esmeralda, Guatapanare and El Morro de Chacopata. *Perna viridis* was later discovered in the northern Caribbean in Kingston Harbour, Jamaica in 1998 (D. Buddo and T. Bowes, pers. comm.). *Perna canaliculus* is

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still geographically isolated, however Carlton (1992) expresses concern that *P. canaliculus* will spread to California where it is now imported daily in large numbers for human consumption. The discovery of introduced populations of *P. perna* and *P. viridis* make the identification of these species based solely on geographic location unworkable.

Since *Perna perna* and *P. viridis* are no longer geographically separated and are difficult to differentiate from each other because of their highly variable morphological characters, other laboratory procedures are necessary to positively identify these species. *Perna viridis* and *P. perna* have been shown to express dimorphism in chromosome numbers and can be distinguished from each other using cytological characters. *Perna viridis* has 15 pairs of homologous chromosomes (Ahmed 1974) and *P. perna* (including introduced Texas populations) has 14 pairs of homologous chromosomes (Ahmed 1974; Jacobi et al. 1990; Holland et al. 1999). Unfortunately, no karyotype analysis is available for *P. canaliculus*. In this report, we confirm the identity of green mussels in Hillsborough Bay as *P. viridis* utilizing both morphometric and cytogenetic characters. We further describe its present well-established distribution throughout and beyond the Tampa Bay estuarine system.

MATERIALS AND METHODS

Initial specimens for identification were collected at the TECO Gannon Station Power Plant in Hillsborough Bay (Fig. 1). Specimens were collected from the screens of the water intake channel by a diver and from inside the intake tunnels during routine maintenance on 15 July 1999. Preserved voucher specimens are deposited in the Division of Invertebrate Zoology, American Museum of Natural History, New York (AMNH 290101), Mote Marine Laboratory (MML 8027.00, 8028.00, 8029.00) and at Lamar University (LU 100399).

Specimens for cytological analyses were collected from the intake boot (flared flange at the base of the water intake pipe) and tunnels of the same power station on 13 October 1999. Chromosome preparations were obtained from gill tissue using a modified colchicine-Giemsa technique (Yaseen 1995; Holland et al. 1999). Specimens were immersed in 0.05% colchicine in aerated 35 ppt seawater at room temperature for 12 h. After this, gill tissues were isolated and placed into a hypotonic solution of 0.8% sodium citrate for 40 min. Tissues were fixed twice in freshly prepared mixtures of 3 parts methanol to 1 part glacial acetic acid for 20 min each, then left in the fixative for 12 h. Cell suspensions were prepared by gently grinding tissues with a microtube pestle in a 1.5 ml microcentrifuge tube containing 60% acetic acid. Disaggregated tissues were then transferred back into fresh fixative by pipette. Cell suspensions were then dropped on cleaned glass slides held at a 45 ° angle from a height of 15 cm. Slides were air-dried then stained with a 24:1 mixture of 0.1 M phosphate buffer (pH 6.8) and Giemsa for 5 min. Photographs were taken of representative mitotic spreads and karyotypes were formed.

Florida Fish and Wildlife Conservation Commission (Florida

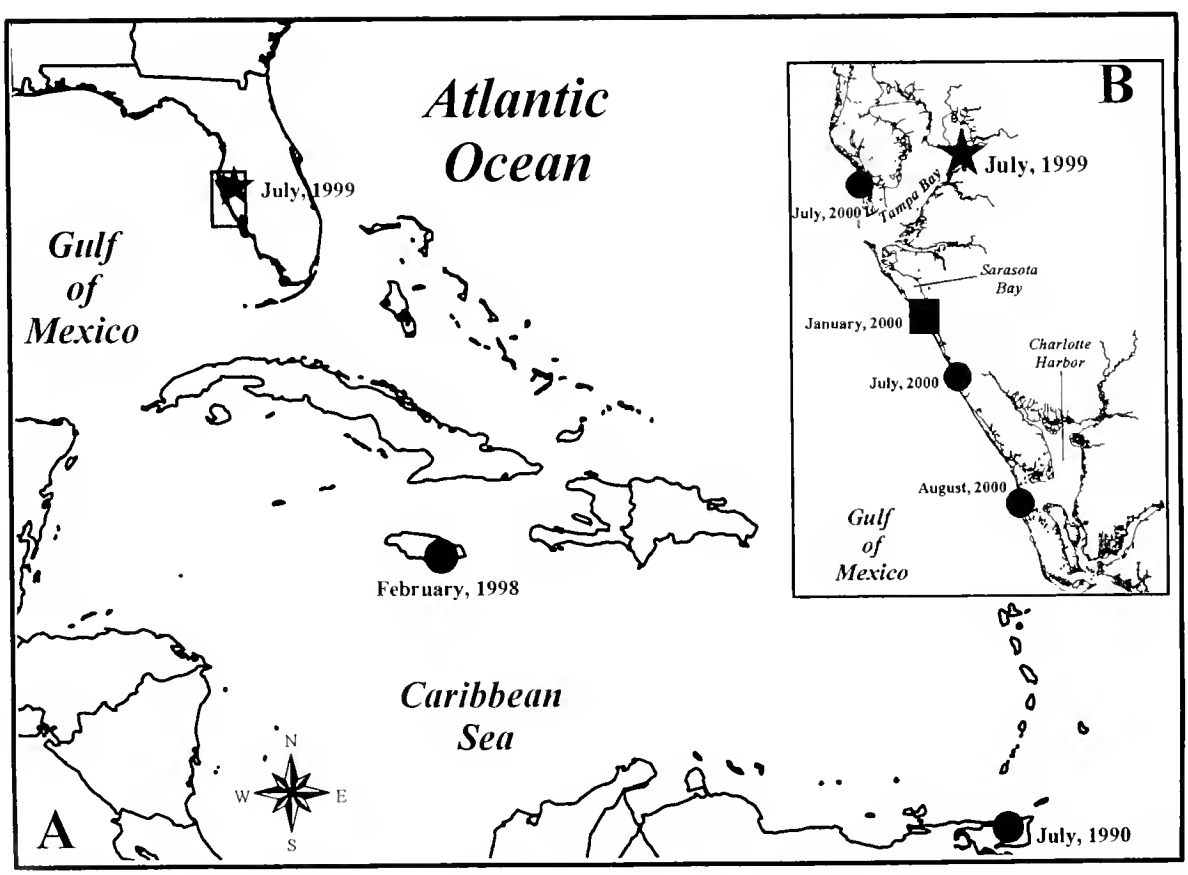

Figure 1. (A) Confirmed sightings of *Perna viridis* in the Caribbean Sea and Gulf of Mexico. (B) Selected documented sightings of *Perna viridis* along the central west coast of Florida.

Marine Research Institute, St. Petersburg, Florida), Mote Marine Laboratory (Sarasota, Florida), U. S. Coast Guard Cutter *Joshua Appleby* (based in St. Petersburg, Florida), Florida Caribbean Science Center (U. S. Geological Survey, Gainesville, Florida), and Great Lakes Science Center (U. S. Geological Survey, Ann Arbor, Michigan) conducted several surface and diving surveys to determine the extent of the introduction of *P. viridis* along the western coast of Florida.

RESULTS

Morphological characters of 26 mature specimens ranging from 24–87 mm in shell length were examined. All specimens have a two-part retractor muscle scar and no anterior adductor muscle scar that is consistent with mussels belonging to the genus *Perna* (mussels belonging to the genus *Mytilus* have a single retractor muscle and an anterior retractor muscle) (Siddall 1980a; Vakily 1989). The specimens were predominantly brilliant green and blue-green sometimes having concentric blue-green bands and the central portion of the shell light brown in some specimens, matching descriptions for *P. viridis* according to Siddall (1980a,b), Vakily (1989), and Dance (1974). Adult shell shape and muscle scars (Fig. 2) corresponded closely to *P. viridis*, as depicted in Siddall's (1980a) comparative review. The mantle margin of preserved and living specimens showed no enlarged sensory papillae, which are characteristic of *P. perna* (Siddall 1980a). Morphological observations of the 26 specimens suggested identification as *P. viridis* but did not conclusively exclude *P. perna*, known to occur along the nearby Gulf coast of Texas.

A diploid number of 2n = 30, characteristic of *Perna viridis*, was scored in 18 mitotic metaphases from two individuals. Qualitative morphological groupings of chromosomes indicated 10 meta-submetacentric and 5 subtelocentric pairs (Fig. 3), consistent with the chromosome morphology for *P. viridis* presented by Ahmed (1974). These results confirmed the tentative morphological identification as *P. viridis*, and indicate the introduction of a second alien pernid mussel to the Gulf of Mexico.

Since its initial discovery at TECO Gannon Station Power Plant in Hillsborough Bay, TECO has reported the presence of *P. viridis* at their Big Bend Station Power Plant in Lower Hillsborough Bay since July 1999 (E. Bietia, pers. comm.); and Florida Power Corporation, Bartow Power Plant on Weedon Island near the entrance to Old Tampa Bay, Florida (27°51′40″N; 82°36′09″W) first noticed these mussels in July 1999 (D. Bruzek, pers. comm.). Range surveys have found living *P. viridis* specimens as far north as John's Pass in St. Petersburg, Pinellas County, Florida, as far south as the Venice Fishing Pier (Venice, Sarasota County, Florida) in the Gulf of Mexico (M.A. Blouin, A. J. Benson, pers. comm.). The largest specimens (many exceeding 100 mm) have been found in Tampa Bay. Specimens found outside of Tampa Bay have been relatively small (1–40 mm). Well-established communities of *P. viridis* have been found in Hillsborough Bay, Old Tampa Bay, Middle Tampa Bay, Lower Tampa Bay, Boca Ciega Bay, and Lower Manatee River (D.C. Marelli, A.J. Benson, M.A. Blouin, C. O'Neil, pers. comm.). The first specimen (10 mm) discovered in Sarasota Bay was found on an invertebrate collecting box in New Pass in January 2000. Four out of eight specimens collected from the Mote Marine Laboratory's New Pass intake pump (19 April 2000) were completely brown (including the inside lip). Over 800 specimens of *P. viridis*, sizes ranging from 0.6–10 mm, were inadvertently collected on a PVC tidal gauge that had been attached to the Apollo Beach Pier for 41 days (29 April to 8 June 2000). Juvenile specimens collected off this tidal gauge which ranged in size from 0.6 to approximately 2.5 mm were golden colored with concentric reddish-brown zigzag markings; specimens greater than 2.5 mm started to develop a green lip; specimens greater than 3 mm showed alternating concentric green banding with the reddish-brown zigzag markings; specimens greater than 4.5 mm started developing their green lip with no banding. Several completely brown juveniles were found.

DISCUSSION

Carlton's 1992 review of introduced marine and estuarine mollusks of the Atlantic, Gulf and Pacific coasts of North America revealed that 36 introduced species have become established. *Perna perna* was the only clearly introduced marine mollusk in the Gulf of Mexico (Carlton 1992). Turgeon et al. (1998) states there are now 47 introduced species of marine and estuarine mollusks in North America: 25 bivalves and 22 gastropods. *Perna viridis* now

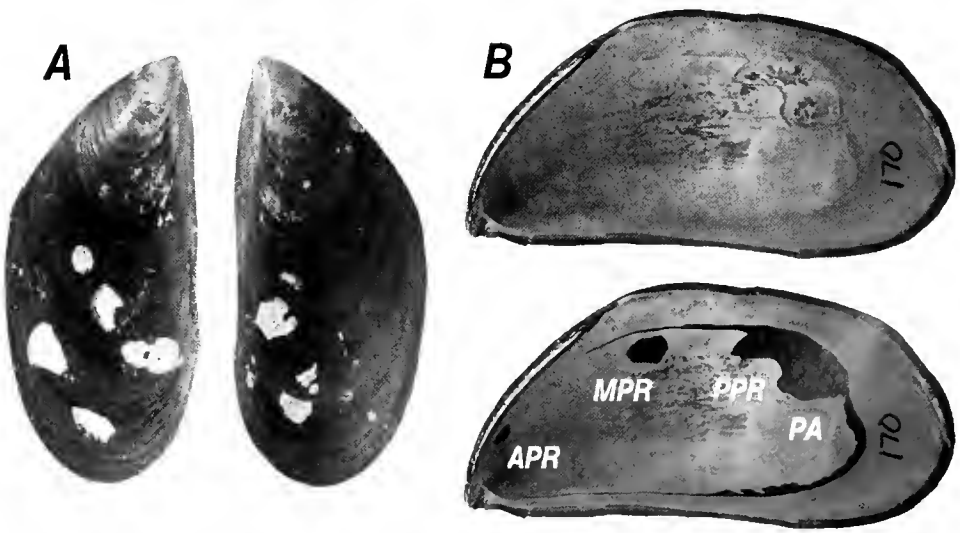

Figure 2. Shell morphology of *Perna viridis* from Tampa Bay, Florida. (A) External view, shell length 87 mm. (B) Internal view of right valve, shell length 104 mm; lower view with enhanced muscle scars and pallial line. APR, anterior pedal retractor muscle; MPR, middle pedal retractor muscle; PPR, posterior pedal retractor muscle; PA, posterior adductor muscle.

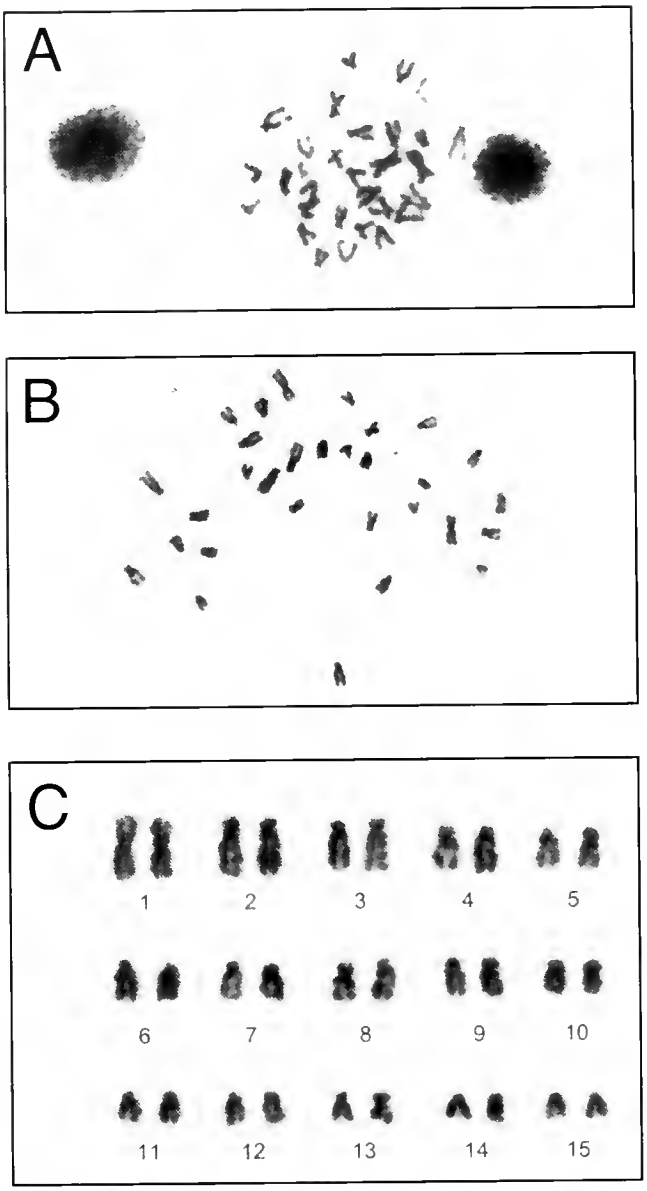

Figure 3. Mitotic chromosomes (A and B) and standard karyotype (C) (from B) prepared from gill tissue from *Perna viridis* specimens collected October 13, 1999 from TECO's Gannon Power Station, Hillsborough Bay (in Tampa Bay), Florida. The 15 pairs of chromosomes are diagnostic for *P. viridis*.

joins these lists as the second introduced mollusk in the Gulf of Mexico and warrants concern as a potential ecological threat to coastal waters of North America (Morton 1997).

Perna viridis shares many characters common to other invasive bivalve species (Morton 1997). For example: *P. viridis* has an extensive capacity for larval and adult dispersal, early maturity, rapid growth rate, and high productivity. *Perna viridis* has also been very successful at finding suitable habitat along the southeastern coast of the Gulf of Mexico.

Extensive Capacity for Dispersal

The planktotrophic larval stages of *Perna viridis* remain free-swimming for 10–20 days (Siddall 1980b, Vakily 1989), during which they can be dispersed over great distances by ocean currents or in the ballast water of ocean-going ships (Carlton 1992). *Perna* species also foul the hulls and anchor chains of transoceanic vessels as byssally-attached adults by which they can be transported internationally (Carlton 1987). Both of these mechanisms were apparent factors in the 1986 introduction and subsequent extraordinarily rapid dispersal of the freshwater zebra mussels (*Dressenia polymorpha*) through the inland waters of North America (Keevin et al. 1992, Claudi & Mackie 1994). Barge and other commercial shipping traffic plying marine/estuarine Intracoastal Waterway (ICWW) interconnecting the Gulf of Mexico and Atlantic states (from the southeastern tip of Texas to Boston, Massachusetts) provide an accessible mechanism for dispersal of *Perna viridis* beyond its present southeastern Gulf of Mexico range.

Early Maturity

Perna viridis becomes sexually mature at 15–30 mm shell length — approximately 2–3 months after fertilization (references in Siddall 1980b; Vakily 1989). In stable tropical environments, spawning can occur year round exhibiting two more-or-less prominent peaks (Vakily 1989; Siddall 1980b; Huang 1985). Spawning can also be easily induced by rapid changes in water temperature (Vakily 1989; Siddall 1980b). Larvae settle within 15-20 days if a suitable substratum (i.e. some type of hard surface — natural or man-made) is present.

Rapid Growth

The combined effects of a number of environmental factors such as water temperature, salinity, currents, food availability, densities and pollution have a considerable effect on the growth rates of *P. viridis* (see Vakily 1989). Morton (1987, p. 163) stated, "Throughout its broad range, *P. viridis* has been reported to have a phenomenal growth rate of some 10 mm per month . . ." Vakily (1989) examined previously published data and compiled growth rates for wild cultures of *P. viridis* ranging from 2.2–10.6 mm per month. Huang et al. (1983) projected an annual growth rate of 60 mm in the relatively polluted waters of Tolo Harbour, Hong Kong. A rough estimate of growth rate of Florida specimens was calculated using the largest specimen (10 mm) collected from the PVC tide gauge at Apollo Beach Pier using shell length/number of days substratum was submerged. This calculation assumes *P. viridis* larvae settled almost immediately, the largest specimen collected was the first to settle, no predation, and a constant growth rate. The estimated growth rate for this specimen was 7.2 mm per month. The following age approximations are based on this growth rate. The specimens collected initially from the TECO Gannon Station Power Plant (15 July 1999) ranged from 24.4–86.6 mm in length, and are estimated to be 3–12 months old, suggesting that initial settlement occurred in July 1998. Some of the largest specimens (over 100 mm) were collected from shipping channel marker buoys in Middle Tampa Bay (14 December 1999); the largest specimen (109.8 mm) is estimated to be over 15 months old suggesting initial settlement occurred in September 1998. The specimens collected on the PVC tide gauge at Apollo Beach (8 June 2000) range in size from 0.6–10 mm and are estimated to be 2–41 days old.

There is considerable fluctuation in growth rates and population densities of all species of *Perna* caused by the combined effects of several environmental factors such as water temperature, food availability [especially chl-a and particulate organic carbon (Rivonkar et al. 1993)], setting densities, currents exposure, and

pollution (Vakily 1989). The highest densities of *P. viridis* in Hong Kong are consistently recorded from Tolo Harbour (4000 adult individuals m^{-2}) and Victoria Harbour (200–246 adult individuals m^{-2}) which are polluted by domestic, agricultural and industrial effluents (Huang et al. 1983). The lowest densities in Hong Kong waters (<100 m^{-2}) are recorded from eastern (exposure to strong wave action), southern (exposure to strong wave action) and western waters (salinities consistently <5 ppt) (Morton 1987). Huang et al. (1983) reported the highest density recorded from Tolo Harbour was 35,000 m^{-2}.

When living in a favorable area, *P. viridis* has been found to spawn year round (reference in Huang et al. 1983). Cheung (1993) studied *P. viridis* population dynamics in the polluted Tolo Harbour, Hong Kong and states (p. 20) "With high densities and a fast growth rate, the productivity of the mussel bed is considerable and is higher than many bivalve populations." Hicks and Tunnell (1995) reported that the closely related *P. perna* could be found forming a distinct mussel belt on the lower mid-intertidal zone of granite jetties in densities of up to 27,200 m^{-2} small individuals (mean = 16 mm). These jetties commonly support 10,000–12,000 individuals m^{-2} (Hicks and Tunnell 1995). Hicks and Tunnell have found on average 25,000–30,000 individuals m^{-2} of *P. perna* spat; the maximum density found was 127,000 m^{-2} (Sea Grant Media News Center website 1999).

Habitat

Tampa Bay provides hard substrata and temperature/salinity regimes similar to those where *P. viridis* naturally occurs in the Indian and southwestern Pacific Oceans as well as areas such as Japan and Venezuela where *P. viridis* has been introduced. It is a subtropical estuary located on the west central coast of peninsular Florida and is the largest open water estuary in the state (Estevez 1989; Lewis and Estevez 1988). It is a Y-shaped embayment, 56 km in length and 16 km wide encompassing over 1000 km^2 (Estevez 1989; Pribble 1999). The average depth of the bay is approximately 4 m. The deepest natural area of Tampa Bay (27 m) is near the mouth of the bay (Pribble 1999). Tampa Bay is also a highly urbanized area and supports many shipping and recreational boating activities. The Port of Tampa is Florida's largest port and consistently ranks among the top 10 ports nationwide in shipping tonnage (Tampa Bay Estuary Program 2000; Meineke 1999). Over 25 million tons of phosphate and related products move through the Port of Tampa annually — more than any other port in the world (Tampa Bay Port Authority 2000). Ships dumped an estimated 543 million gallons of ballast water into Tampa Bay in 1996 (Pittman 1999) from around the world, thus providing an excellent transport mechanism for *P. viridis*.

Perna viridis has been able to maintain dense populations in Tampa Bay and is spreading along the southeastern Gulf of Mexico shore and estuaries despite the lack of continuous, natural, rocky intertidal habitats just as *P. perna* has done on the southwestern Gulf of Mexico shore since its introduction to Texas in 1990. *Perna viridis* can be found attached to hard substrata such as rock faces, wharf piles, among algal holdfasts, and mangrove roots. In deeper waters, where hard substrata are not available, initial attachment may be to sediment grains, shell fragments or other mussels resulting in mussel beds on sandy or muddy bottoms (Agard et al. 1992; Hickman 1991; Jeffs et al. 1999; Berry 1978). Disjunct man-made structures including pier pilings, channel markers, buoys, jetties and bulkheads provide excellent surfaces for *Perna* larvae to settle and grow on. Structures like these are common along the southeastern Gulf of Mexico.

Perna viridis prefer temperatures from 31–32 °C in the summer and 26–28 °C in the winter (Vakily 1989; Agard et al. 1992). Nearly linear growth was evident when temperatures were from 23.6–30.8 °C and cessation of growth occurred when winter temperatures were from 17–18 °C in Tolo Harbour, Hong Kong (Cheung 1993). The low (6 °C) and high (37.5 °C) lethal temperatures for *P. viridis* were recently determined through laboratory experiments (Segnini de Bravo et al. 1998). Water temperatures range from 11–32 °C in subtidal areas of Tampa Bay and vary more widely in intertidal areas (Lewis & Estevez 1988). Maximum water temperatures of 28–30 °C are found from June through August; minimum temperatures of 15–18 °C occur from December through February (Pribble 1999).

Perna viridis prefers salinities 27–33 ppt although laboratory tests showed 50% survival rate after two weeks of exposure to 24 and 80 ppt (reference in Vakily 1989; Agard et al 1992). The low (0 ppt) and high (64 ppt) lethal salinities for *P. viridis* were recently determined through laboratory experiments (Segnini de Bravo et al. 1998). *Perna viridis* can also survive extended exposures to salinities as low as 20 ppt that can occur after a heavy rain (Agard et al. 1992). As would be expected, salinity varies throughout Tampa Bay — higher salinities are found near the mouth of the bay in areas that interact strongly with the Gulf of Mexico; lower salinities are found in areas affected by the inflow of fresh water from rivers and occur in regions farthest away from the Gulf of Mexico (Pribble 1999). Maximum salinities, up to 38 ppt usually occur in June (Pribble 1999). Lowest salinities, down to 15 ppt usually occur in September (Pribble 1999). The rapid dispersal of *P. viridis*, its high thermohaline tolerance and establishment of successful colonies in the southern Caribbean Sea and Tampa Bay suggest that it will continue to spread in the Gulf of Mexico.

SUMMARY

Rayl (1999, p. 6) states: "Experts estimate that aquatic invasive species cost the United States at least $2 billion every year, including fisheries losses, removal and recovery efforts, and sport fishing losses — and the figure is projected to rise." Considering the reproductive rate, growth rate of *Perna viridis* and the similarity in environmental conditions between the southwestern coast of Florida and this mussel's natural habitats, area scientists, resource managers, and others are concerned about its potential impact to native communities as well as coastal economics. Introduced aquatic species frequently cause massive transformation of the structure and function of native ecosystems. Like other introduced species, *P. viridis* has the biotic potential to flourish and to out-compete native species. Economically, the presence of dense mussel colonies on water intake systems, channel markers and buoys, threatens to increase the frequency of maintenance activities and their associated costs. The capacity of *P. viridis* to attach byssally to hard surfaces makes it a major macrofouling threat to industrial facilities drawing raw seawater for operations (Rajagopal et al. 1995). *Perna viridis* is already creating serious operational problems for many power plants and other water intake systems around the world such as Madras Atomic Power Station in southern India (Rajagopal et al. 1991) and Jamaica Private Power Company (T. Bowes, pers. comm.). Byssal mats that are formed by dense population of *P. viridis* are already a problem for local power plants. These byssal mats (and all that grows in them) are

ripped off the hard substratum by the water current flowing to the condensers. The inflowing water pushes the mats against the small pipes in the end of the condensers and the mats act like a clogged air-conditioning filter. This causes pumps to work harder, overheat and potentially cause plant shut down (E. Beitia and D. Bruzek, pers. comm.). In April 2000, *P. viridis* was found growing inside one of the water intake pumps at Mote Marine Laboratory and caused the pump to overheat requiring the pump to be shut down decreasing the amount of water available for daily operations (C. Traxler, pers. comm.). Heavy colonies could also potentially sink channel marker buoys, creating a hazard to navigation as was seen in Texas with *P. perna* (Hicks pers. comm.).

All of the news may not be bad. All three *Perna* species are a valuable food source high in protein and are harvested from wild population or aquaculture facilities for human consumption (Vakily 1989). In 1955 the first commercial mussel farm started in the Philippines (Vakily 1989). Thailand (*P. viridis*) and New Zealand (*P. canaliculus*) are the only two other countries where *Perna* is being cultured on an intensive commercial scale (references in Vakily 1989). India (*P. viridis*) is the only country in Asia with a substantial mussel industry not based on culture; over 3,000 tonnes are harvested annually (Vakily 1989). Rafts, racks, bamboo poles, date-palm stakes, iron bars, ropes, rope-webs and longlines are used to culture *Perna* (Vakily 1989). Mussel seed are rarely produced artificially but are collected on spat collectors during peak spawning seasons (Vakily 1989). Aquaculture of *P. canaliculus* started about 20 years ago and has continued to expand, with production almost doubling in the last 4 years to reach 70,000 tonnes y^{-1} (Jeffs et al. 1999). *Perna canaliculus*, marketed as Greenshell™ mussel, is internationally recognized as a premium-eating mussel and is thought to possess unique remedial properties in rheumatoid and osteoarthritis (references in Jeffs et al. 1999). The success of *P. viridis* in the southeastern Gulf of Mexico, the demand for these mussels in the food industry and potential biomedical importance could spawn new aquaculture opportunities.

In rapid response to the discovery of *Perna viridis* in Tampa Bay, staff at the Florida Fish and Wildlife Conservation Commission (at the Florida Marine Research Institute, St. Petersburg) and Mote Marine Laboratory (Sarasota) have issued public announcements in the printed and audio media, as well as on the World Wide Web (http://www.mote.org~debi/perna.phtml, http://www.fmri.usf.edu/invert/pviridis.htm) to inform the public and solicit information about the spread of the mussel (Associated Press, 1999; Pittman, 1999; Roberts, 1999; Florida Fish and Wildlife Conservation Commission, 2000). Meeting presentations and posters have been given (Ingrao et al. 1999; Benson et al. 1999; Ingrao et al. 2000) and live-animal displays and requests for further information were put in place at the publicly available Mote Marine Aquarium. In spite of the activity surrounding this newly recorded introduction, the mussel is already well established in its new environment and its eradication is not expected. Clearly, priorities must be directed at monitoring its ecological and economic impacts, and at developing methods for its control and use.

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ESTABLISHMENT OF THE GREEN MUSSEL, *PERNA VIRIDIS* (LINNAEUS 1758) (MOLLUSCA: MYTILIDAE) ON THE WEST COAST OF FLORIDA[†]

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ABSTRACT In 1999, the green mussel, *Perna viridis*, was first observed in Tampa Bay, Florida. This was the first reported occurrence of this Indo-Pacific marine bivalve in North America. The mussels found in Tampa Bay were confirmed to be *P. viridis* based on both morphological and genetic characteristics. Since the initial discovery, surveys in Tampa Bay and on the west coast of Florida have documented the growth, recruitment, and range expansion of *P. viridis*. From November 1999 to July 2000, the mean shell length of a Tampa Bay population increased from 49.0 mm to 94.1 mm, an increase of 97%. Populations of *P. viridis* are successfully reproducing in Tampa Bay. Recruitment was observed on sampling plates in May and continued through July 2000. The full extent of mussel colonization is not clear, but mussels were found outside Tampa Bay in St. Petersburg, Florida, south to Venice. Based on these studies it is evident that *P. viridis* has successfully invaded Tampa Bay and the west coast of Florida. The long-term impact of *P. viridis* on native communities off the west coast of Florida cannot be predicted at this time.

KEY WORDS: *Perna viridis*, introductions, green mussel, Florida, Gulf of Mexico

INTRODUCTION

Florida's ecosystems have been and continue to be the recipients of many new nonindigenous species introductions and invasions (U.S. Congress 1993, Carlton & Ruckelshaus 1997). Tampa Bay, located on the west coast of central Florida, has been particularly open to exotic species introductions because of its temperate to subtropical climate and active industrial, commercial, and recreational use. In August of 1999, the Tampa Electric Company reported finding an unknown bivalve fouling the surfaces in their cooling water intake tunnels at both their Gannon and Big Bend power stations in and near the city of Tampa, Florida (G. Shofner, personal communication). They were identified as *Perna viridis* (Linnaeus 1758) based on their karyotype (Ingrao et al. 2001). This was the first report of this species in North America. Nonindigenous species are potentially invasive when they display some of the following characteristics: high fecundity, short generation time, long-lived, high dispersal rate, phenotypic plasticity, broad native range, abundant in native range, and tolerant of a wide range of conditions (Williams & Meffe 1998).

Perna viridis is native to the coastal marine waters of the Indo-Pacific region including the Persian Gulf to the South China Sea (Siddall 1980, Vakily 1989). It is a byssally attached, sessile bivalve naturally inhabiting estuarine waters where the salinity ranges from 27-33 PSU, although it tolerates salinities as low as 16 PSU (Sundaram & Shafee 1989). The optimal annual temperature range for *P. viridis* is 26–32°C; survival rate was 50% at 10° and 35°C after two weeks of exposure (Sivalingam 1977). *Perna viridis* exhibits a wide tolerance for sub-optimal environmental conditions, tolerating large fluctuations in sediment flux and organic enrichment (Morton 1985, Lee 1986). Like other marine mussels, *P. viridis* attaches to hard substrates, but is also capable of colonizing soft substrates (Agard et al. 1992). *Perna viridis* generally occur at depths from the surface to 10 m, although settlement of juvenile mussels has been reported greatest at depths of between 2 and 3 meters (Tan 1975, Cheong and Chen 1980). This species is a suspension feeder selectively feeding primarily on algae (Sivalingam 1977, Yap et al. 1979). It has been intensively cultured within its native range as a protein source for human consumption and been shipped to many islands in the South Pacific over the last several decades for this purpose (Chalermwat et al. 1988, Eldredge 1994).

Perna viridis is a dioecious broadcast spawner (Sivalingam 1977) displaying a wide variation in spawning pattern within its native range, encompassing unimodal, bimodal, and asynchronous modes (Walter 1982, Lee 1986, Tuaychareon 1991, Rajagopal et al. 1998b). Following fertilization, zygotes develop into free-swimming veligers within 16 hours, and umbonate larvae develop at about 20 hours post fertilization (Sivalingam 1977). Larvae remain free-swimming for 2–3 weeks, then settle onto hard substrata and byssally attach (Vakily 1989, Tuaychareon 1991).

There are currently three species of *Perna* (*P. perna* (Linnaeus 1758), *P. canaliculus* (Gmelin 1791), and *P. viridis*). They inhabit tropical and subtropical coastal marine waters worldwide; however, none occur naturally in North America (Siddall 1980). Within the past decade, both *Perna perna* (Hicks & Tunnel 1993) and *Perna viridis* (Ingrao et al. 2001) have been introduced into the Gulf of Mexico and southern Caribbean (Agard et al. 1992, Rylander et al. 1996, D. Buddo, personal communication). The known distribution of *Perna viridis* in the western hemisphere includes the following countries: Venezuela, Trinidad, Jamaica, and peninsular Florida in the United States (Fig. 1). While *P. perna*, the edible brown mussel, is native to the southern Caribbean, *P. viridis* is not, and until the recent introductions of *P. perna* and *P. viridis* into the Gulf of Mexico and southern Caribbean the species did not occur sympatrically (Siddall 1980). *Perna canaliculus*, known as the greenshell mussel, is native to waters surrounding New Zealand. Shell color, morphology, and especially geographic location have been used in the past to identify and distinguish among the species of *Perna* (Siddall 1980). Although color has been used as a distinguishing characteristic, it is not a definitive feature. Shells of young *P. viridis* mussels are a brilliant green while the adults are dark green to brown, and young *P. perna*

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Figure 1. Shaded circles represent *P. viridis* populations along coastlines.

observed in Texas can be green but are brown as adults (D. Hicks, personal communication). *Perna viridis* can be distinguished from *P. perna* by examining the karyotype of somatic cells: *P. perna* has 28 chromosomes while *P. canaliculus* and *P. viridis* have 30 (Ahmed 1974, Holland et al. 1999, Ingrao et al. 2001). Therefore, because color and morphological characters are unreliable for distinguishing between the species in this genus (Siddall 1980), more defining characteristics are needed to determine the identity among species of the genus *Perna*.

METHODS

Study Sites

From November 17–19, 1999, biologists from the U.S. Geological Survey (USGS) and the Florida Marine Research Institute (FMRI) sampled bridge pilings, buoys, channel markers and shorelines of Tampa Bay. The northern section of the Bay consists of two smaller bays, Hillsborough and Old Tampa bays. Because the two power plants that had reported finding *Perna viridis* were located in Hillsborough Bay, we decided to sample Old Tampa Bay to determine the species' range within the entire Tampa Bay system. Green mussels were collected by hand off bridge pilings from just above the water surface to approximately one-half meter below the surface. In addition, we qualitatively surveyed two bridges in Old Tampa Bay using scuba divers in late February 2000. Divers also attempted to locate additional populations of mussels near the mouth of Tampa Bay from March through July 2000. In the latter part of July, we expanded our survey along the west coast of Florida to further document the extent of their range. We began surveying coastal waters south of Tampa Bay at Venice and proceeded in a northerly direction to Johns Pass at the north end of Treasure Island. In addition to direct observation, we received reports of *P. viridis* observations from private citizens, governmental agencies and other organizations, which were helpful in locating other populations.

Approximately thirty adult *P. viridis* were collected from the wooden fenders beneath the Howard Frankland Bridge (Interstate 275) over Old Tampa Bay during late February 2000 and monthly thereafter. Mussels were fixed in 5% seawater formalin and preserved in 70% ethanol for future analysis of reproductive condition. These mussels were examined morphologically and used to determine the mean length of adults in the Old Tampa Bay population. In addition, we established recruitment collectors in the same vicinity. Recruitment collectors consisted of three 15 cm × 15 cm unglazed quarry tiles attached to a stainless steel frame which were protected from predation by a section of 1 cm × 1 cm polypropylene mesh. Tiles were replaced monthly and exposed tiles were taken to the laboratory for examination using a magnifier lamp as well as a dissecting microscope. All *P. viridis* recruits settling on the tile surface were counted in the laboratory. Scrapings from pier and bridge pilings were also examined in the laboratory to determine the identity of any commensal organisms.

Identification by Genetic Characterization

Isolation of Total DNA from Bivalves

DNA was purified from specimens of *P. perna* (Port Mansfield, Texas), *P. viridis* (Pelican Island, Trinidad), *P. canaliculus* (Marlborough Sound and Castlepoint, New Zealand), and *Perna* species collected from Tampa Bay, Florida. Whole animals were collected, preserved in 95% ethanol or isopropyl alcohol, and shipped by express mail to the laboratory. Total DNA was purified from adductor muscle tissue (*ca.* 0.5–0.14 g) as previously described (Frischer et al. 2000b). This protocol routinely yielded from 30 to 800 µg DNA/g tissue of high molecular weight genomic DNA suitable for PCR amplification depending on the source of tissue.

PCR Amplification of the Mitochondrial Cytochrome C Oxidase Subunit I Gene

A 713-bp fragment of the mitochondrial cytochrome c oxidase subunit I gene (COI) was amplified using primers LCO1490 (5′- ggt caa caa atc ata aag ata ttg t) and HCO2198 (5′- taa act tca ggg tga cca aaa aat ca) from each of the mussel species (Baldwin et al. 1996). Primers were synthesized using an ABI DNA/RNA synthesizer (model 394) by the Molecular Genetics Facility at the University of Georgia. Amplification was accomplished using the Qiagen *Taq* PCR Master Mix System following the standard protocol recommended by the vendor (Qiagen, Valencia, Calif.) except that to each 25 µl reaction, 0.05 µl of T4 Gene Protein (Roche Molecular Biochemicals, Indianapolis, IN) was added to enhance amplification. Amplification conditions were as described by Baldwin et al. (1996); 35 amplification cycles (95°C, 1 min; 54°C, 1 min; 72°C, 1 min) initiated by a 3-min denaturation step at 95°C and followed by a 10-min extension step at 72°C.

Sequencing

To facilitate sequencing of the PCR amplified COI gene fragment from the Tampa Bay *Perna* specimen, *P. perna*, and *P. viridis*, and *P. canaliculus* specimens, the COI PCR product was cloned into the bacterial plasmid sequencing vector PCR 2.1 TOPO cloning vector using the TOPO™ TA cloning system following the instructions provided by the manufacturer (Invitrogen, Carlsbad, Calif.). Sequences were determined by automated sequencing at the Molecular Genetics Facility (University of Georgia) using an ABI automated sequencer (models 373 and 377) as previously described (Frischer et al. 2000b). The complete COI amplicon sequence was obtained using the plasmid vector targeted (M13) sequencing primers M13-20-F (5′- tgt aaa acg acg gcc agt) and M13-48R (5′- agc gga taa caa ttt cac aca gga). Complete sequences were assembled using the assembly and editing features of the DNAsis software package version 7.00 (Hitachi Software Engineering Co.).

Phylogenetic Analysis

Sequences were aligned relative to each other and several other COI fragments from other mollusks available in GenBank using the CLUSTAL W version 1.7 multiple sequence alignment algorithm (Thompson et al. 1994). Alignments were viewed and edited using the Genetic Database Editor (GDE) (Smith et al. 1992). Percent sequence similarity between organisms was determined using the sequence alignment procedure available in the DNAsis software package version 7.00 (Hitachi Software Engineering Co.). Phylogenetic trees were inferred and drawn using the TREECON for Windows software package version 1.3b (Van de Peer & De Wachter 1994, 1997) using the Kimura two-parameter model for inferring evolutionary distance. Bootstrap estimates (100 replicates) of confidence intervals were also made using the algorithms in TREECON.

RESULTS

The identity of the *Perna* species collected in Tampa Bay was confirmed to be the green mussel, *Perna viridis*, based on both morphological and genetic characteristics.

Morphological Characteristics

The presence and location of muscles and muscle scars have previously been reported to be useful identifying characteristics for the genus *Perna* (Siddall 1980). The lack of an anterior adductor muscle confirms that the mussel in Tampa Bay is a species of *Perna*. Furthermore, the position of the anterior component of the posterior retractor muscles appears most similar to that of *P. viridis*. Externally, the most obvious characteristic of the mussels collected in Tampa Bay is that both juveniles and adults are a bright green to dark green color (Fig. 2). Although shell color is a poor identifying characteristic for these bivalves, to our knowledge there have been no reports of *Perna perna* that were green as adults. Due to the ambiguities associated with the identification of *Perna* species based solely on morphological and coloration characteristics, specimens of three *Perna* species were genetically compared with the mussels found in Tampa Bay.

Genetic characteristics

An identically sized 713 bp fragment of the mitochondrial encoded cytochrome c oxidase subunit gene was amplified, cloned, and sequenced from each *Perna* species studied. These sequences have been deposited in GenBank. Accession numbers are AF298850, AF298851, AF308731 and AF298852 for *P. perna* (Texas), *P. viridis* (Trinidad), *P. canaliculus* (New Zealand) and *P. viridis* (Tampa Bay), respectively. Phylogenetic comparison between *Perna* species and *Perna* species collected from Tampa Bay indicated that the Tampa Bay specimens were most closely related to *P. viridis* (Fig. 3). The sequence similarity between *P. perna*, *P. viridis*, *P. canaliculus*, and the Tampa Bay species are shown in Table 1. Sequence similarity between *Perna* species ranged from 78.2% to 99.5% with the highest similarity between the Tampa

Figure 2. The green mussel, *Perna viridis*, collected from Tampa Bay, Florida, November 1999.

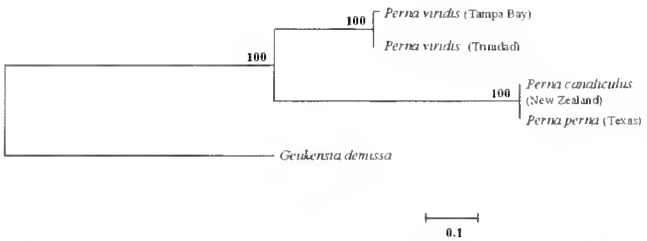

Figure 3. Inferred taxonomic relationship between *P. viridis* in Tampa Bay and other *Perna* species. The phylogenetic tree was derived from nucleotide sequence comparison of a 713 bp fragment of the mitochondrial cytochrome c oxidase subunit I gene (mtCOI). The tree was artificially rooted with the mtCOI sequence from the ribbed mussel (*Geukensia demissa*) retrieved from GenBank (U56844). The scale bar indicates 0.1 fixed nucleotide substitutions per site. Numbers refer to bootstrap values (from 100) for each node.

Bay specimens and the *P. viridis* specimens collected from Trinidad. These results confirm that the *Perna* species currently in Tampa Bay, Florida, is *Perna viridis*.

Distribution, Growth, and Reproduction in Tampa Bay Region

Since the initial discovery of *P. viridis* at two electric power plants in Tampa Bay, Florida, populations have been documented throughout the bay and extending into the Gulf of Mexico. Through July 2000, green mussels have been documented from numerous locations in Tampa Bay (including Hillsborough and Old Tampa bays), Sarasota Bay, and in the Gulf of Mexico from Venice, north to Treasure Island at Johns Pass (Fig. 4). One week after our final survey, mussels were discovered south of Venice at Boca Grande, a barrier island in the Gulf of Mexico just outside of Charlotte Harbor (M. Blouin, personal communication). Mussels were collected at the northernmost and southernmost sites in the final survey; therefore, the full extent of their geographic range along the west coast of Florida is still to be determined.

Mussels collected from the Gannon Power Station (Hillsborough Bay) in October 1999 ranged in shell length from 17.9 to 30.1 mm (mean = 23.6 mm). These animals were reproductively mature and even the smallest individuals began to spawn when they were taken into the laboratory. *Perna viridis* has been reported to achieve sexual maturity in two to three months at a shell length of approximately 20 mm (Vakily 1989, Tnaychareon 1991). The mussels collected in November 1999 from the population of *P. viridis* on the Howard Frankland Bridge (Old Tampa Bay) had a mean shell length of 49.0 mm. Subsequent collections from this population demonstrate rapid growth as the mean length of mussels increased nearly 92% to 94.1 mm in July 2000 (Fig. 5). Growth rates have been reported from a number of areas within the native range. *Perna viridis* in the nutrient rich Hong Kong Harbour grew to a length of 50 mm in the first year, to 75 mm in the second year, and to 85 mm in the third year (Cheung 1993). In a power plant intake structure in India, mussels grew to a length of 27 mm in 49 days and 119 mm in 375 days (Rajagopal et al. 1998a). The highest growth rate recorded is from Singapore at 10.6 mm per month during the first year of culture (Cheong and Chen 1980). According to Vakily (1989), it is difficult to define maximum size and longevity of members of this genus.

Perna viridis recruits were first detected in May 2000 on recruitment collectors placed near the Howard Frankland Bridge in Old Tampa Bay. Small mussels (<10 mm shell length) were prominent on both the collectors and bridge structures during June and July (Fig. 6). Cheung (1993) documented two recruitment periods per year in a Hong Kong harbor, the first from July to September, and the second from November to March.

Tampa Bay community

Tampa Bay is a large estuary approximately 57 km in length and 20 km in width. The entire Tampa Bay watershed encompasses 6,739 km^2 with 967 km^2 of open water. Carlton (1996) views much of the cosmopolitan species that make up the Florida marine biota as cryptogenic. Because it is probable that introductions to Florida by European settlers have occurred for five centuries, it is difficult to differentiate native from exotic species in Florida (Carlton and Ruckelshaus 1997). Commensal organisms, endemic or cryptogenic, found during our surveys to occur with *P. viridis* include other species of bivalves, crustaceans, barnacles, bryozoans, cnidarians, and annelids (Table 2). Though our samples do not represent the entire biota in the bay, we consider them representative of the community in which *Perna viridis* is now established in Tampa Bay.

TABLE 1.

Percent sequence similarity (above diagonal) and the number of nucleotide differences between the Tampa Bay *Perna* species, other *Perna* type species, and the ribbed mussel *Geukensia demissa* mitochondrial cytochrome c oxidase subunit I gene.

	G. demi	*P. perna*	*P. viridis*	*P. can*	TB sp.
G. demi	100	60.5	60.9	65.2	60.7
P. perna	243/614	100	79.1	99.5	78.2
P. viridis	240/614	129/615	100	79.3	99.2
P. can	402/617	615/618	489/617	100	78.4
TB sp.	241/614	134/615	5/617	484/617	100

Genbank accession numbers and abbreviations: *G. demi* (U56844, *Geukensia demissa*); *P. perna* (AF298850, *Perna perna*); *P. viridis* (AF298851, *Perna viridis*); *P. can* (AF308731, *Perna canaliculus*); TB sp. (AF298852, Tampa Bay *Perna* species).

TABLE 2.

A list of commensal invertebrates present from samples collected off bridge pilings of the Courtney Campbell Parkway over Old Tampa Bay. Identification confirmed by Jon Fajans, University of Florida.

Phylum	Species
Mollusca	*Crassostrea virginica* (Linneaus, 1758)
	Ischadium recurvum (Rafinesque, 1820)
	Brachiodontes exustus (Linneaus, 1758)
	Crepidula fornicata (Linneaus, 1758)
	Crepidula plana Say, 1822
Arthropoda	*Tanystylum orbiculare* Wilson, 1878
	Caprella penantis Leach, 1814
	Jassa falcata (Montagu, 1808)
	Petrolisthes galathinus (Bosc, 1802)
	Balanus amphitrite Darwin, 1854
	Balanus improvisus Darwin, 1854
	Balanus eburneus Gould, 1841
Bryozoa	*Bowerbankia gracilis* (O'Donoghue, 1926)
	Membranipora arborescens (Canu & Bassler, 1928)
Cnidaria	*Bouganvillia sp.*
	Campanularia sp.
Annelida	*Nereididae*

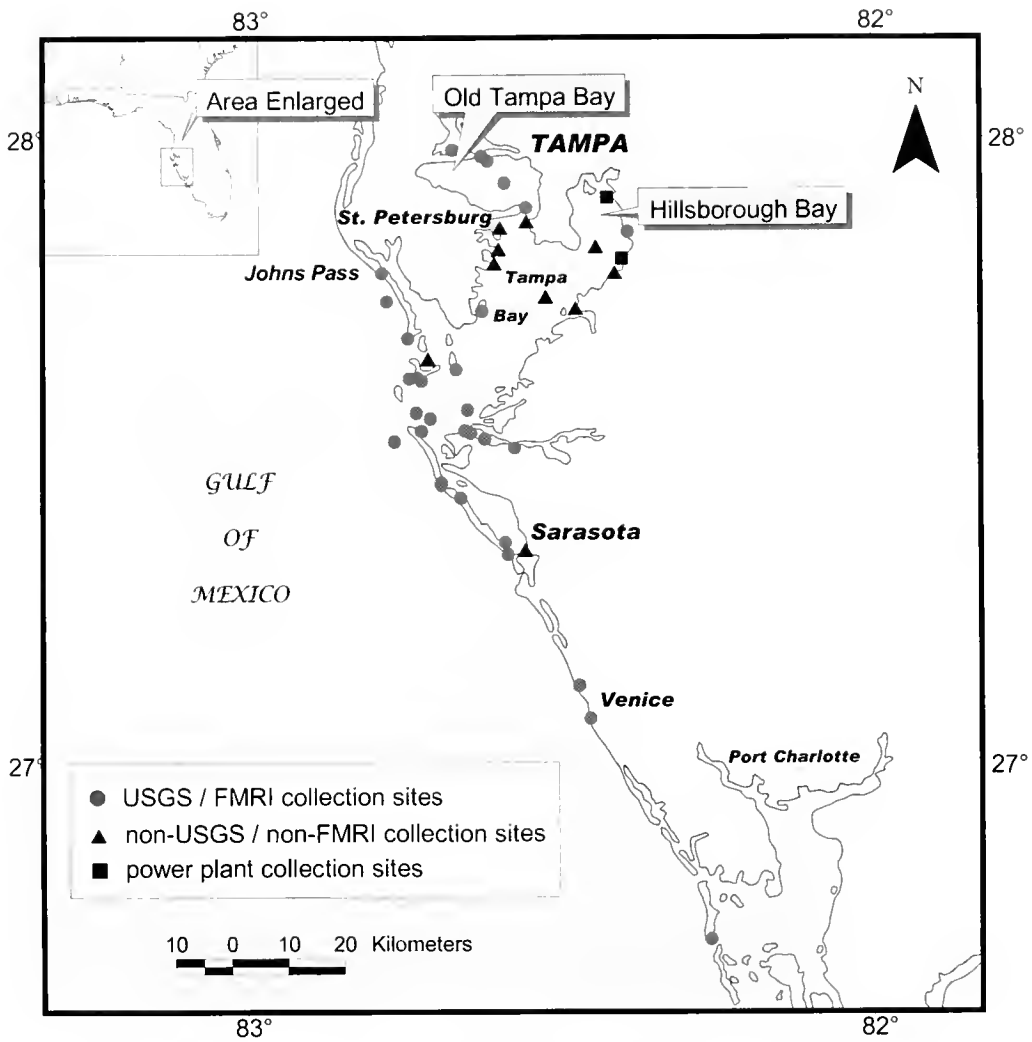

Figure 4. Locations of *Perna viridis* collected or observed, August 1999 to August 2000.

DISCUSSION

The results of this study indicate that the mitochondrial COI gene can be used to genetically identify *Perna* individuals to the species level. Sequence comparison of amplified mitochondrial COI gene products indicated that the *Perna* species discovered in Tampa Bay, Florida is *Perna viridis*. This confirms the reported presence of *Perna viridis* in the Gulf of Mexico that was based on cytogenetic karotyping (Ingrao et al. 2001). Furthermore, these studies suggest that molecular comparison of the mitochondrial COI gene can provide a diagnostic molecular marker for identifying *Perna* at the species level. However, despite the high similarity between the Tampa Bay and Caribbean *Perna viridis* specimens, five nucleotide differences between the two mussels were observed. The presence of these sequence differences suggests that COI sequences may contain sufficient variation for detecting intrapopulation variation within *Perna viridis* populations. Additional sequencing studies will be needed to test this hypothesis. Further studies will also be required to evaluate whether the COI locus will be useful for larval identification techniques (Frischer et al. 2000b, Bell & Grassle 1998, Baldwin et al. 1996). Interestingly, the similarity between *P. perna* and *P. canaliculus* was extremely high (95.5%) suggesting that these two species may be ecomorphs rather than true species. However, since these results were derived from a single specimen of *P. perna* and seven *P. canaliculus*, additional systematic analysis will be required to confirm this conclusion.

Green mussels were initially identified from Hillsborough Bay in August 1999 at electric power generating stations. However, green mussels were not limited to the vicinity of the power stations and furthermore, larger mussels were found in Old Tampa Bay suggesting that it was the initial colonization site for green mussels in Tampa Bay. Thousands of barnacle-encrusted green mussels were discovered on the bridge pilings there indicating that the mussels had been present for an extended period of time. Although we did not quantify the populations on each bridge piling, it was visually apparent that the number of green mussels on each piling decreased as we sampled from the center of the bridge to the shore. We observed no mussels along the shoreline, which consisted of suitable substrate such as rip-rap, concrete retaining walls, and mangrove prop roots. Most of the mussels were observed subtidally during our surveys of the bridge pilings. However, a smaller

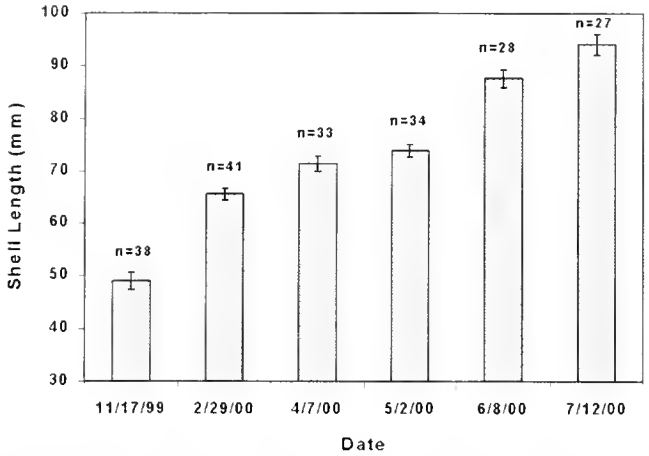

Figure 5. Mean shell length of *Perna viridis* individuals collected from Old Tampa Bay, 1999-2000. Data include means plus and minus one standard error.

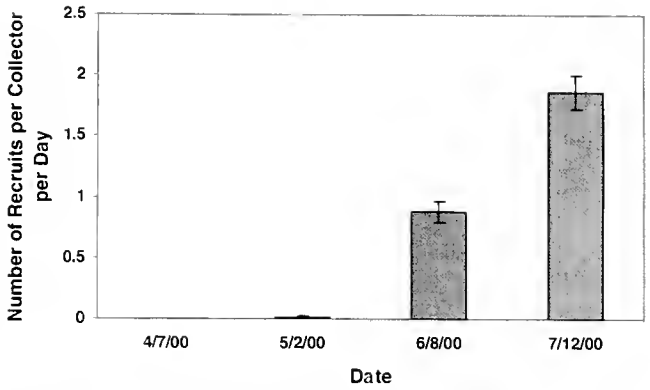

Figure 6. Mean recruitment of *Perna viridis* to collector plates in Old Tampa Bay, Florida, 2000. Data include number of recruits per collector per day plus and minus one standard error.

portion were observed intertidally where they were exposed to atmospheric conditions for short periods.

At the sample sites along the Gulf coast from Venice to Treasure Island (Johns Pass), mussels were smaller and fewer when compared to the sites sampled in 1999 from Old Tampa Bay (A. Benson, personal observation). However, mussels were common enough to be detected on bridge and dock pilings and channel markers after just several minutes of diving. Having observed a visible decrease in the number and size of mussels in our surveys outside of Tampa Bay reaffirmed that the Port of Tampa was the initial site of this introduction from which they subsequently were transported by currents out of the Bay along the coast. We did not observe green mussels in slow-moving backwater areas in the Tampa Bay estuary or along the Gulf coast and Intracoastal Waterway.

Perna viridis is the most recent nonindigenous mollusk to enter Florida, probably introduced into Tampa Bay as larvae entrained in ship ballast water. Ballast water transport has become a major source for the introduction of marine organisms (Carlton and Geller 1993, Carlton and Ruckelshaus 1997). Other modes of introduction such as accidental releases by the seafood or aquaculture industries, or purposeful release of mussels seem far less likely. Although originally from the Indo-Pacific region, we suspect that the source of introduction of green mussels into Tampa Bay was the southern Caribbean region. Just over 40% of the ships arriving in the port of Tampa "in ballast" (carrying significant quantities of water ballast) originate in the Gulf of Mexico or Caribbean, and less than 1% originate in areas even remotely close to the native range of *P. viridis* (Carlton et al. 1995). The spread of *P. viridis* to other areas in Florida and the southeast United States can be expected and will be limited only by the availability of transport vectors and the physiological tolerance of the mussels. Users of industrial water such as power plants may use mechanical or chemical control methods to reduce or eliminate mussels. Efforts to limit future introductions of nonindigenous species must include assessments of risk to native habitats, risk from species or communities in ports of origin, and the risks of potential vectors of introduction (Carlton et al. 1995, Carlton and Ruckelshaus 1997, Simberloff et al. 1997). Concurrently, measures must be developed to prevent such introductions.

Our data and observations demonstrate that *Perna viridis* is able to grow, over-winter, and successfully reproduce in Tampa Bay. In the period since its introduction, estimated to be about two years, the green mussel has colonized mostly man-made habitats throughout the estuary and has entered the Gulf of Mexico where currents have begun transporting larvae south along the Gulf Coast. The rapid growth and maturity, high fecundity, high dispersal rate, and obvious ability for *P. viridis* to thrive in the Tampa Bay estuary indicate that this species is already becoming invasive as defined by Williams and Meffe (1998). As mentioned earlier, *P. viridis* is a tropical species where normal water temperatures range from 26°C to 32°C. Based on water temperatures in Tampa Bay since 1998, green mussels have not yet experienced water temperatures below the LC_{50} of 10°C, but water temperatures have been as low as 12.3°C, much colder than waters in their native range. Historic data as far back as 1974 revealed winter water temperatures were below 10°C only in 1977. If green mussels persist in the Bay, cool temperatures may be selecting for a more cold-tolerant population, increasing the potential for northward expansion. Certainly other water quality parameters may be factors in the establishment of the green mussel elsewhere and deserve further investigation.

We expect *P. viridis* to continue to spread south along the Gulf Coast of Florida, possibly to include the Florida Keys and other Caribbean islands (Fig. 7). Based on surface water temperature data, it is likely that green mussels could survive almost anywhere along the Gulf of Mexico coastline, from Florida to Texas and Mexico. *Perna viridis* may survive in less than optimal water temperatures as far north in the Atlantic Ocean as Charleston, South Carolina. If green mussels were able to survive in suboptimal water temperatures in the United States, occasional below-normal winter air temperatures may lower water temperatures enough to kill or limit populations. We might also expect them to survive along the Hawaiian Islands coasts in the Pacific Ocean and along the coast of Mexico but probably not as far north as California in the United States. We must also be aware of the fact that thermal pollution can provide overwintering refugia in temperate climates such as in Japan, resulting in an even greater distribution in North America.

The biological and economic impacts of *Perna viridis* on the Tampa Bay region are difficult to predict. Areas currently colonized by green mussels are human created substrates and not of

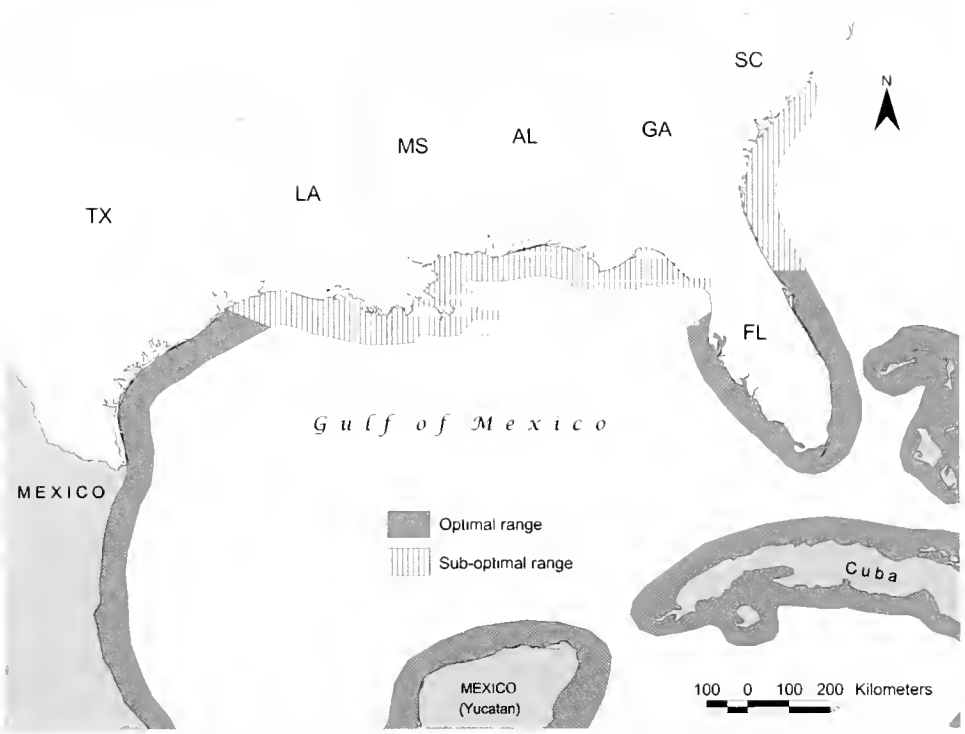

Figure 7. Predicted range of *Perna viridis* in the Gulf of Mexico and western Atlantic waters based on near-surface water temperatures. The lower limit of the sub-optimal range based on LC_{50} of 10°C with two weeks exposure.

particular concern as far as issues of biodiversity are concerned. We are concerned that *P. viridis* will eventually colonize prop roots of the red mangrove, *Rhizophora mangle*, and affect the diversity of the native communities associated with this habitat. Green mussels have already become established on red mangrove prop roots in Trinidad within the past decade (Agard et al. 1992) and more recently in Jamaica (D. Buddo, personal communication). They may also affect the dynamics of the phytoplankton assemblage and increase water clarity in Tampa Bay through active suspension feeding, much as *Potamocorbula amurensis* has in San Francisco Bay and *Dreissena polymorpha* has in numerous freshwater bodies (Alpine and Cloern 1992; Stoermer et al. 1996). However, it is expected that many of the nutrients will be cycled into the benthic community as feces and pseudofeces, possibly causing local shifts in benthic community dynamics (Tsuchiya 1980; Dobson and Mackie 1998, Frischer et al. 2000a).

Impacts are expected to include fouling of unprotected boats and in-water structures such as docks, seawalls, and aids to navigation and interference with cooling water intakes in electric power generating stations. Oddly, green mussels are biofoulers of power plants in their native India (Nair & Murugan 1991; Rajagopal et al. 1995). They are also a major part of the biofouling community in China where they are notorious for fouling navigation buoys with biomasses of as much as 72 kg/m^2 (Yan et al. 1994). *Perna viridis* is also a new member of the biofouling community in Japan. Winter water temperatures normally decimate populations there; however, overwintering of mussels does occur in Tokyo near warmwater discharges (Umemori and Horikoshi 1991). Tampa Bay already has an active fouling community consisting mainly of barnacles, tunicates, and native bivalve mollusks, but none of these animals approaches either the size or the potential accumulated mass of *Perna viridis*. Local electric utilities are already experiencing problems associated with green mussel settlement in intake structures (D. Marelli, personal observation) and it is anticipated that the cost of maintenance in these structures, as well as on navigational buoys and vessels will increase substantially as the *P. viridis* population continues to increase.

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SPECIES COMPOSITION OF BLUE MUSSEL POPULATIONS IN THE NORTHEASTERN GULF OF MAINE

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ABSTRACT We examined the species composition of eleven blue mussel populations in eastern and central Maine, USA using a set of PCR-based genetic markers. Previous reports suggested that mussel populations in the Gulf of Maine were composed of only a single species, *Mytilus edulis*. In contrast, our results clearly indicate that the range of a congener, *M. trossulus*, extends well into the Gulf. The two blue mussels are sympatric in eastern Maine populations, including all of those we sampled within Cobscook Bay, ME. The frequency of *M. trossulus*, however, declines dramatically in the vicinity of Little Machias Bay, ME, so that populations along the coast of central Maine are composed predominantly of *M. edulis* mussels. Among populations containing a mixture of *M. edulis* and *M. trossulus*-specific alleles we observed a low but significant frequency of mussels with hybrid genotypes including putative backcross genotypes, indicating the potential for introgression between these two species. We suggest that larval supply and recruitment, thermal tolerance and perhaps the interplay of these factors likely delimit the southern extent of the range of *M. trossulus* and influence the species composition of blue mussel populations in the northwest Atlantic.

KEY WORDS: blue mussels, *Mytilus* spp., hybridization, biogeography, molecular markers

INTRODUCTION

Mussels in the genus *Mytilus* are widely distributed in the temperate and sub-polar zones of both the northern and southern hemispheres (Gosling 1992a) and are often dominant members of the rocky intertidal community. This genus includes a group of morphologically similar smooth shelled or blue mussels belonging to the *Mytilus edulis* species complex. Blue mussels have been the focus of a wide variety of studies on topics ranging from physiology, biochemistry, ecology, biogeography and pollution ecology, and are economically important as a source of food in many parts of the world (Gosling 1992a). Due to the lack of reliable morphological characters, the taxonomy of blue mussels has been highly confused historically. Based on the work of McDonald et al. (1991), however, who employed a combination of allozyme electrophoresis and multivariate analysis of morphological characters there are currently three recognized species of blue mussel worldwide, *M. edulis* (Linnaeus 1758), *M. galloprovincialis* (Lamark 1819), and *M. trossulus* (Gould 1850). In the northwest Atlantic, allozyme surveys have further indicated that *M. edulis* occurs from Cape Hatteras, NC to the Canadian Maritimes while *M. trossulus* has been observed at multiple locations in southeastern Nova Scotia and eastern Newfoundland, often in mixed populations with *M.edulis* (Koehn et al. 1976, 1984, Varvio et al. 1988, Bates and Innes 1995). Based on these surveys, it has been generally assumed that mussel populations within the Gulf are composed entirely of *M. edulis*.

The species composition of blue mussel populations in the northeast Gulf of Maine, however, had not been examined until Hennigar et al. (1996) used allozyme electrophoresis to assay variation at the mannose phosphate isomerase (*Mpi*) locus for mussels sampled from several sites in the Gulf, including the Bay of Fundy. The *Mpi* allozyme locus is considered to be the most diagnostic enzyme marker for identifying *M. edulis* and *M. trossulus* (e.g. Mallet & Carver 1995, Comesaña et al. 1999). Hennigar et al. (1996) observed a high frequency of *M. trossulus*-specific *Mpi* alleles at Digby, Nova Scotia and Hospital Island, New Brunswick, providing the first indication that the range of *M. trossulus* extends into the Gulf of Maine.

The presence of *M. trossulus* in this region is a serious concern to the Maine mussel culture industry. Although the mussel fishery in Maine has traditionally relied on harvesting from both natural beds and bottom culture leases seeded with juvenile mussels, the industry has recently begun to employ suspended culture systems. Reports from mussel farms in the Maritime Provinces of Canada suggest that *M. trossulus* mussels contain less meat than *M. edulis* mussels and their shells are prone to fracturing during processing (Freeman et al. 1994, Mallet and Carver 1995). Given species-specific growth rates, survival, and production losses, Mallet and Carver (1995) have estimated that, under raft culture conditions, the economic value of *M. edulis* is 1.7 times that of *M. trossulus*.

Blue mussels also figure prominently in environmental monitoring efforts within the Gulf of Maine. The Gulfwatch program, initiated in 1991 by the Gulf of Maine Council on the Marine Environment, assesses coastal habitat exposure to inorganic and organic contaminants by examining the accumulation of heavy metals, PCBs, and other toxins in mussels collected from 60 sites located between southern Massachusetts and Nova Scotia (Sowles et al. 1997). Significant differences have been observed in the seasonal patterns of lead, chromium, zinc, and mercury accumulation between *M. edulis* and *M. trossulus* (Mucklow 1996) emphasizing the need for accurate species identification and consideration of taxonomic affinity when interpreting data from the Gulfwatch program (Lobel et al. 1990, Sowles et al. 1997).

In this study, we set out to determine the southern-most extent of *M. trossulus*' range in the Gulf of Maine. Using a set of diagnostic molecular markers, we have assessed the species composition of eleven blue mussel populations sampled from central to eastern Maine. Recently, Bates and Innes (1995) and Comesaña et al. (1999) have used allozyme electrophoresis as well as DNA-based genetic markers to investigate the degree of hybridization between these two species and test hypotheses regarding the ecological factors which affect their distribution on the coast of Newfoundland. Although our study was not designed to specifically test for associations between species abundance and ecological factors, we compare our results to their findings to assess whether similar ecological and genetic mechanisms may be shaping the local distribution of and interaction between *M. edulis* and *M. trossulus* throughout the region where they are sympatric.

MATERIALS AND METHODS

Approximately 30 to 50 adult blue mussels (10.9–61.8 mm shell length) were collected from eleven locations along the coast of central and eastern Maine, including two separate sites near the Darling Marine Center (see Fig. 1). Sampling was conducted between February and July of 1999 at the lowest tide level during spring tides. Mussels from all twelve locations were transported live to the University of Maine were they were dissected. DNA was isolated from a tissue biopsy using the protocol of Schizas et al. (1997), wherein a small piece of mantle tissue (~10 mg) from each mussel was digested with 40 ng of proteinase K in 20 μl of a buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100 at 55°C for 3 hours. After the digestion process, the samples were heated to 95°C for 15 minutes and 20 μl of Gene Releaser (BioVentures Corp.) was added. The resulting slurry was incubated at 65°C for 30 s, 8°C for 30 s, 65°C for 90 s, 97°C for 180 s, 8°C for 60 s, 65°C for 180 s, 97°C for 60 s, 65°C for 60 s and centrifuged at 13,000 rpm for 2 min. The supernatant containing total cellular DNA was then removed to a new microcentrifuge tube.

Isolated DNA was used as template in each of four polymerase chain reaction (PCR)-based genetic assays. The application of the mt16S-F, Glu-5′, and ITS markers followed the protocols provided by Rawson and Hilbish (1995), Rawson et al. (1996a), and Heath et al. (1995), respectively. In this study, the use of the *Mytilus* anonymous locus 1 (MAL-1) marker essentially followed the protocol in Rawson et al. (1996b) except that the oligonucleotide primers were redesigned to amplify a smaller PCR product. Three of these markers, ITS, MAL-1, and Glu-5′, target the nuclear genome while the mt16S-F marker targets the mussel female mitochondrial DNA lineage. For each assay, 50 ng of template DNA was added to a 12.5 μl reaction (total volume) containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 2.5 nmol dNTPs, 1 U *Taq* DNA polymerase (Promega) and 50 pmol of assay-specific forward and reverse oligonucleotide primers (Table 1). The reactions were initially denatured for three minutes at 94°C and then incubated for 30 cycles at 94°C for 30 s, followed by an assay-specific annealing temperature (see Table 1) for 30 s, and 72°C for 1 minute. The ITS, MAL-1 and mt16s-F markers relied on PCR to generate equal length PCR products for both *M. edulis* and *M. trossulus*. When these products were then digested with the appropriate restriction enzymes (see Table 1) species-specific restriction fragment length patterns were generated. For the Glu-5′ marker, which is similar to that designed by Inoue et al. (1995), PCR amplification produced *M. edulis* and *M. trossulus*-specific products that differ significantly in size (see Rawson et al. 1996a for details). For each marker the species-specific PCR products or PCR/RFLP fragments were resolved on 1.5% to 2.5% agarose gels.

Individual genotypes and haplotypes were scored as outlined for ITS in Heath et al. (1995), Glu-5′ in Rawson et al. (1996a), and mt16S-F in Rawson and Hilbish (1995, 1998). Although Rawson

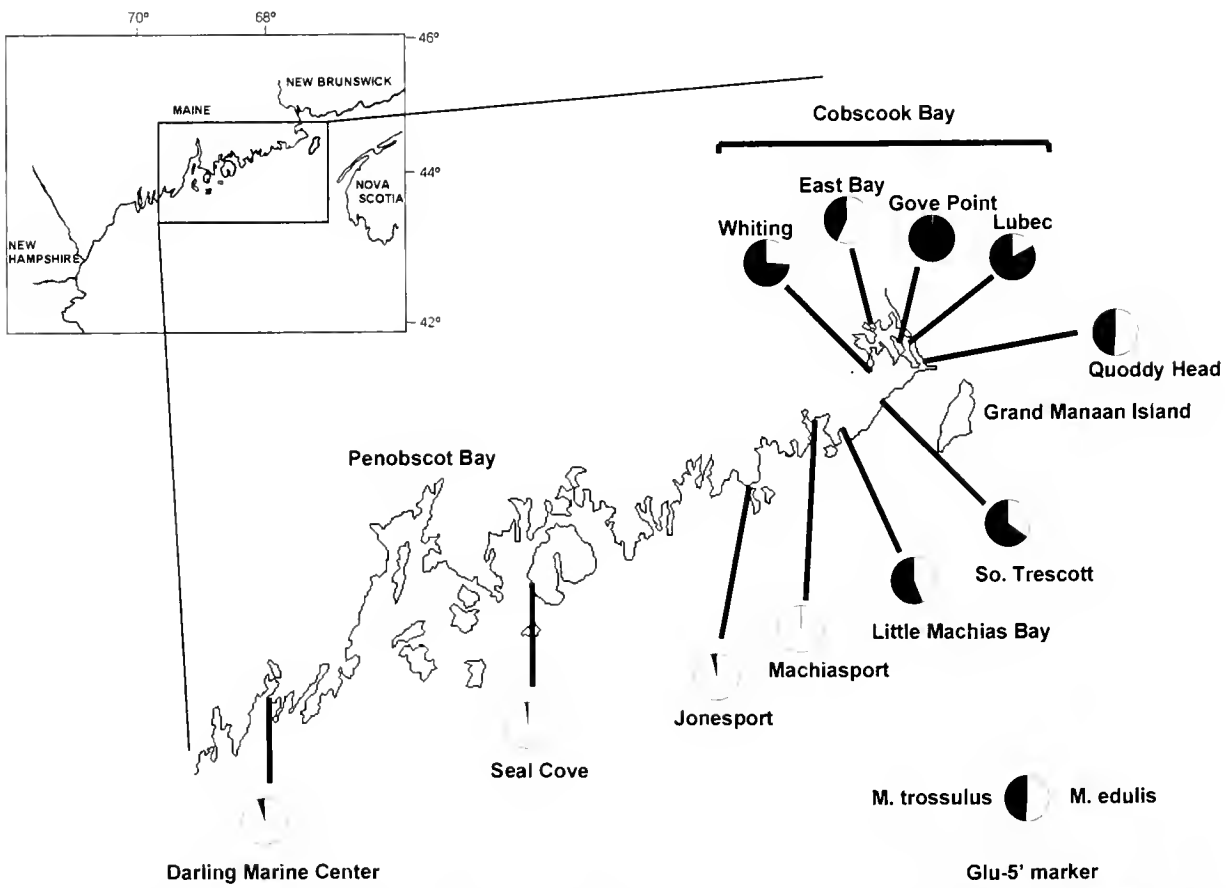

Figure 1. Map of the blue mussel populations in central and eastern Maine sampled in this study indicating the frequency of *Mytilus edulis* and *M. trossulus*-specific Glu-5′ alleles observed at each location. The frequency of Glu-5′ alleles at the two Darling Marine Center sites were combined for clarity.

TABLE 1.

Oligonucleotide primer sequences, annealing temperature, and restriction enzymes used in each of the four DNA-based markers employed in this study.

Marker	Primer Sequence	Temperature	Enzyme
ITS	Forward: GTTTCCGTAGGTGAACCTG Reverse: CTCGTCTGATCTGAGGTCG	50°C	*Hhal*
Glu-5′	Forward: GTAGGAACAAAGCATGAACCA Reverse: GGGGGGATAAGTTTTCTTAGG	57°C	NA
MAL-1	Forward: GAAGCGTATTTGGTCACTGGCAC Reverse: GTCATAAAATGGAACATCTGAGTC	50°C	*SpeI*
mt16S-F	Forward: CCGGTCTGAACTCAGATCACGT Reverse: CTGCCCAGTCGAACTAGAGTAAT	47°C	*SpeI* + *EcoRV*

et al. (1996b) described the application of the MAL-I marker for distinguishing *M. trossulus* and *M. galloprovincialis*, their study did not include *M. edulis*. As they reported, when MAL-I PCR products generated from *M. trossulus* DNA are digested with the restriction enzyme *SpeI* two different restriction profiles are observed. In contrast, when MAL-I PCR products generated from *M. edulis* DNA are digested with the same restriction enzyme, a single profile distinct from those observed for *M. trossulus* results (Fig. 2). Allopatric populations of *M. edulis* (Lewes, DE and Cape Anne, MA) and *M. trossulus* (Port Orford and Newport, OR) are fixed for alternate PCR/RFLP profiles at all four of these DNA-based markers (Table 2).

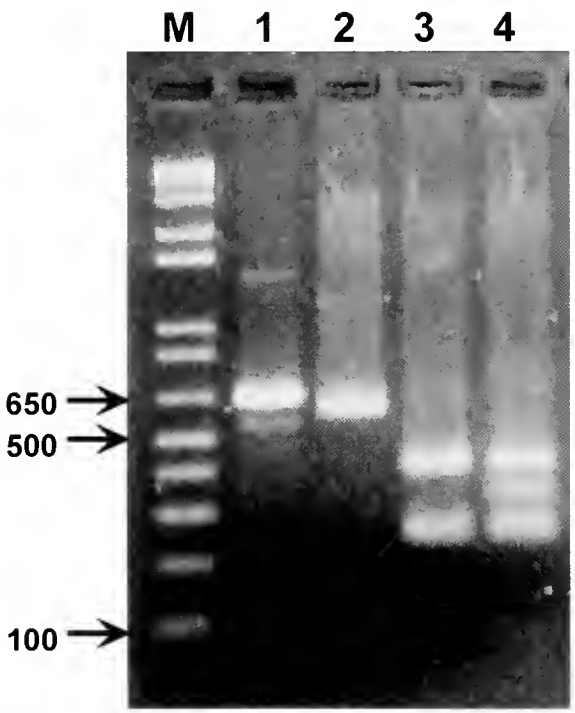

Figure 2. Restriction fragment length polymorphism for *M. edulis* and *M. trossulus* at the MAL-I locus analyzed on a 2.5% agarose gel. PCR amplification produces a single ~650 base pair band for both species as shown in the lane 1. Digestion of the PCR product produces a single banding pattern for *M. edulis* (lane 2) and two separate patterns for *M. trossulus*. Lane 3 shows the RFLP pattern for an individual homozygous for the T1 allele while lane 4 was produced by an individual heterozygous for the T1 and T2 alleles.

Allele and haplotype frequencies were estimated for each of the twelve sampling locations. Post hoc tests of heterogeneity were conducted using the allele frequencies at each locus to determine whether there were significant changes in the frequency of *M. trossulus*-specific alleles across all eleven study sites as well as across subsets of sites in eastern and central Maine. For each test, an R × C contingency table was constructed and an exact test for heterogeneity in allele frequency performed using the STRUC program distributed with the GENEPOP software package (Raymond and Rousset 1995). To estimate the frequency of mussels with multi-locus *M. trossulus*, *M. edulis* and hybrid genotypes, a hybrid index was constructed based on the number of *M. trossulus*-specific alleles a given individual carried at the ITS, MAL-I, and Glu-5′ markers. Thus, mussels homozygous for *M. trossulus* alleles at each nuclear marker received a score of 6, those homozygous for *M. edulis* alleles received a score of 0, while mussels with mixed multi-locus genotypes received a score between 1 and 5. For example, mussels which were heterozygous at two loci and homozygous for *M. trossulus*-specific alleles at the third locus received a score of 4. The frequency of mussels falling into each hybrid index score was estimated for the Whiting, East Bay, Gove Point, Lubec, Quoddy Head, South Trescott and Little Machias Bay samples combined. Each of these samples contained *M. trossulus* alleles at a frequency in excess of 5%.

RESULTS

We successfully genotyped 518 individual adult mussels (*Mytilus* spp.) for three, and in most cases, four PCR-based genetic markers. There was highly significant spatial heterogeneity in the frequency of *M. trossulus* and *M. edulis*-specific alleles and haplotypes across the 11 locations sampled in this study (Table 2). This was illustrated by the variation in the frequency of *M. trossulus* and *M. edulis* Glu-5′ alleles shown in Figure 1. In mussel populations east of the Little Machias Bay site, including all of the sites sampled in Cobscook Bay, *M. trossulus* alleles and haplotypes were quite common, with frequencies ranging from a low of 34.3% at South Trescott to a maximum of 97.4% at Gove Point. West of Little Machias Bay, however, the frequency of *M. trossulus*-specific alleles declined precipitously. There was no evidence of spatial heterogeneity in allele frequency for any of the four markers we employed among the Machiasport, Jonesport, Seal Cove, and both of the Darling Marine Center populations (Table 2) indicating that populations along the central coast of Maine consistently contained predominantly *M. edulis* alleles.

The observed spatial variation for each of the four markers was

TABLE 2.

Allele frequencies and haplotype frequencies in reference mussel populations and at twelve sites sampled in Gulf of Maine.

	ITS[a]			Glu-5′[a]			MAL-1[a]				mt16S-F[b]		
Location	n	E	T	n	E	T	n	E	T1	T2	n	A	B
Allopatric Populations													
Newport, OR	52	0.0	100.0	52	0.0	100.0	52	0.0	71.0	29.0	52	0.0	100.0
Port Orford, OR	27	0.0	100.0	27	0.0	100.0	27	0.0	74.1	25.9	27	0.0	100.0
Lewes, DE	40	100.0	0.0	40	100.0	0.0	40	100.0	0.0	0.0	40	100.0	0.0
Cape Anne, MA	36	100.0	0.0	36	100.0	0.0	36	100.0	0.0	0.0	36	100.0	0.0
Eastern Maine													
Whiting	82	25.6	74.4	82	28.0	72.0	76	21.1	55.3	23.6	41	22.0	78.0
East Bay	74	54.0	46.0	82	57.3	42.7	80	55.0	36.3	8.7	42	54.7	45.3
Gove Point	74	1.4	98.6	76	2.6	97.4	72	1.4	65.3	33.3	37	0.0	100.0
Lubec	90	16.7	83.3	94	21.3	78.7	86	16.3	53.5	30.2	47	14.9	85.1
Quoddy Head	114	49.1	50.9	114	54.4	45.6	112	50.0	29.5	20.5	57	49.1	50.9
South Trescott	68	64.7	35.3	70	65.7	34.3	68	61.8	27.9	10.3	35	62.9	37.1
Little Machias Bay	118	55.9	44.1	120	56.7	43.3	118	54.2	33.1	12.7	60	55.0	45.0
Central Maine													
Machiasport	74	100.0	0.0	94	100.0	0	70	100.0	0.0	0.0	46	100.0	0.0
Jonesport	94	95.7	4.3	96	95.8	4.2	84	95.2	4.8	0.0	47	95.7	4.3
Seal Cove	58	97.3	2.7	62	100.0	0	60	100.0	0.0	0.0	30	100.0	0.0
Darling Center A	48	91.6	8.4	42	90.5	9.5	34	94.1	5.9	0.0	26	92.3	7.7
Darling Center B	76	97.3	2.7	88	97.7	2.3	82	97.6	2.4	0.0	46	97.8	2.2
Tests for Heterogeneity													
All Sites		***			***				***			***	
Eastern Maine[c]		***			***				***			***	
Central Maine[c]		NS			NS				NS			NS	

[a] Genotypes at the ITS and Glu-5′ markers were scored as in Heath et al. (1995) and Rawson et al. (1996a) and for MAL-1 marker as described in the text. E = *M. edulis*-specific alleles and T = *M. trossulus*-specific alleles.

[b] Haplotypes at mt16S-F were scored as in Rawson and Hilbish (1995, 1998). A = *M. edulis*-specific haplotype and B = *M. trossulus*-specific haplotype.

[c] Tests for heterogeneity in allele/halpotype frequency were conducted across all sites as well as across sites within two regions, Eastern Maine (Whiting, East Bay, Gove Point, Lubec, Quoddy Head, South Trescott, and Little Machias Bay) and Central Maine (Machiasport, Jonesport, Seal Cove, Darling Center A and B).

*** $p < 0.001$.

NS not significant.

highly concordant (Table 2). This was further reflected by the predominance of mussels homozygous for *M. edulis* or *M. trossulus* alleles at all three nuclear loci. For each of the mixed populations containing appreciable frequencies of *M. trossulus* alleles (i.e. Whiting, East Bay, Gove Point, Lubec, Quoddy Head, South Trescott and Little Machias Bay), a hybrid index was calculated for each mussel based on the number of *M. trossulus*-specific alleles at each nuclear marker. Among the 320 mussels sampled from these seven sites, the vast majority (>87%) had index scores of 0 or 6, i.e. nearly all of the mussels sampled were homozygous for all three nuclear makers (Fig. 3). Less than 1% of the mussels we sampled had index scores of 3 and no mussel heterozygous at all three nuclear markers was observed. Of the remaining mussels, most (9%) had index scores of 4 or 5 indicating that these mussels carried more *M. trossulus*-specific than *M. edulis*-specific alleles. In addition, there was a high concordance between multi-locus nuclear genotypes and mitochondrial haplotypes. Of the 117 mussels with an index score of 0, all but one carried the A mt16s-F haplotype fixed in allopatric populations of *M. edulis*, while 161 of 163 mussels with an index score of 6 had the B mt16S-F haplotype found in allopatric populations of *M. trossulus*.

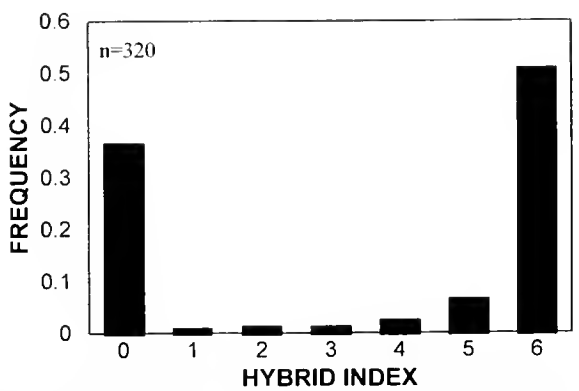

Figure 3. Frequency of mussels with multi-locus hybrid index scores based on the number of *M. trossulus*-specific alleles at each of three nuclear markers. A score of 0 indicates mussels homozygous for *M. edulis*-specific alleles at all three nuclear loci, a score of 6 indicates mussels homozygous for *M. trossulus*-specific alleles and scores of 1–5 indicate mussels containing a mixture of *M. edulis* and *M. trossulus* alleles. The analysis includes only mussels from the seven populations which contained >5% *M. trossulus*-specific alleles.

DISCUSSION

Our results indicate that a marked transition in the species composition of blue mussel populations occurs in the northeastern

Gulf of Maine. This contrasts with the conclusions of Koehn et al. (1976, 1984), who suggested that Gulf of Maine populations were composed solely of *M. edulis* mussels. Their studies, however, did not sample mussel populations between Bar Harbor in central Maine and Halifax on the eastern side of the Nova Scotian peninsula. Together with the results of Hennigar et al. (1996), who found high frequencies of *M. trossulus*-specific *Mpi* alleles in neighboring Passamaquoddy Bay, New Brunswick and Digby, Nova Scotia, our observation of fairly high frequencies of *M. trossulus* at several sites in eastern Maine, especially in the Cobscook Bay region, clearly indicates that mussels with *M. trossulus* alleles occur throughout the Bay of Fundy region and well into the Gulf of Maine.

Our results also suggest that there has been a limited amount of introgression between *M. edulis* and *M. trossulus*. Within populations containing appreciable frequencies of both *M. edulis* and *M. trossulus* alleles in eastern Maine, the vast majority of mussels had multi-locus genotypes consistent with their being pure *M. edulis* or *M. edulis* mussels. Among these multi-locus homozygotes (n = 280), all but three had species-specific mitochondrial haplotypes that were in agreement with their nuclear genotypes. For mussels with multi-locus hybrid genotypes, we found most to have hybrid index scores of 4 or 5, genotypes which are indicative of backcrosses formed by the mating of F1 hybrids with *M. trossulus* individuals. Thus, hybridization between *M. edulis* and *M. trossulus* to produce F1 mussels must occur, at least occasionally, even though we observed no tri-locus heterozygous mussels in our samples and the observation of putative backcross genotypes suggests that a low level of introgression occurs between these species.

A thorough analysis of introgression would benefit from additional nuclear markers (e.g. Boecklen and Howard 1997) and a more rigorous statistical framework (e.g. Nason & Elstrand 1993). From the reports presented to date, however, hybridization between *M. edulis* and *M. trossulus* appears to be relatively limited. Estimates of the frequency of hybrids ranges from a low of <2% (Mallet & Carver 1995) to as much as 23% in Nova Scotia (Saavedra et al. 1996) and 26% in Newfoundland (Comesaña et al. 1999). This level of hybridization is comparable to that reported by Rawson et al. (1999), Sarver and Foltz (1993) and Suchanek et al. (1997) for *M. trossulus* and *M. galloprovincialis* on the Pacific coast of North America. In contrast, several studies have reported a frequency of hybrids in excess of 50 to 80% at locations in Western Europe where *M. galloprovincialis* hybridizes with *M. edulis* (Sanjuan et al. 1994, Comesaña & Sanjuan 1997, Hilbish et al. 1994, Gardner 1996). Although many of these studies have employed different marker systems with varying ability to discriminate hybrid classes, taken together these observations are consistent with phylogenetic studies which suggest that *M. edulis* and *M. galloprovincialis* are sister taxa and *M. trossulus* is more distantly related to these other two taxa (Rawson & Hilbish 1998).

Gardner (1996) has proposed that differential adaptation to environmental conditions is the primary force determining the distribution of all three species of blue mussel, *M. edulis*, *M. galloprovincialis*, and *M. trossulus*, in the Northern Hemisphere. For example, within the most extensively studied blue mussel hybrid zone, several studies have shown that habitat-specific selection based on differential growth or physiology, strength of attachment to the substrate and other factors, plays a major role in the distribution of mussel genotypes across the region of sympatry between *M. edulis* and *M. galloprovincialis* in western Europe (e.g. Hilbish et al. 1994, Comesaña and Sanjuan 1997, see review by Gosling 1992b). Among the other blue mussel hybrid zones studied to date, the importance of differential adaptation is less well documented. Sarver and Foltz (1993) observed a correlation between local temperature and salinity and the distribution of *M. galloprovincialis* and *M. trossulus* on the Pacific coast of North America. Rawson et al. (1999), however, pointed out that hybridization between these two species occurs between Monterey and Cape Mendocino, California, a region that is characterized by extremely complex hydrodynamic conditions, so that limited dispersal may also be involved in determining the distribution of parental and hybrid mussels.

Likewise, in the northwest Atlantic it is presently unclear what mechanisms operate to structure the species composition of blue mussel populations. It has been suggested that *M. trossulus* may be better adapted to estuarine conditions and therefore is more tolerant of low salinity than is *M. edulis* (e.g. Gosling 1992b). Nearly all of the sites we sampled, including those with high frequencies of *M. trossulus* alleles, however, experience oceanic salinities (35 ppt) year round. The only exception was the Machias Bay population which was far enough upriver to occasionally receive pulses of fresh water. Yet, mussels at this site were fixed for *M. edulis* alleles and haplotypes. These results suggest that salinity cannot be the sole selective agent regulating the local distribution of *M. trossulus* and *M. edulis*. Bates and Innes (1995) and Comesaña et al. (1999) have also investigated the role of wave exposure in structuring mussel populations off the coast of Newfoundland. Their studies failed to find a clear association between the frequency of *M. trossulus* and degree of wave exposure in the populations they sampled. Similarly, we observed high frequencies of both *M. edulis* and *M. trossulus* alleles in all populations sampled in eastern Maine, including relatively sheltered sites within Cobscook Bay as well as the exposed sites on the outer coast such as Quoddy Head and South Trescott. Thus, neither salinity nor wave exposure is likely to explain the precipitous decline in the frequency of *M. trossulus* that we have observed in eastern Maine.

The spatial variation in the frequency of *M. trossulus* mussels that we observed among eastern Maine populations may reflect variation in larval supply and recruitment. Johnson and Black (1982) and Watts et al. (1990) suggested that genetic patchiness among populations of *Siphonaria sp.* and *Echinometra mathaei* were likely due to stochastic variation in larval recruitment. On a broader scale, regional current patterns can potentially limit larval dispersal and promote genetic divergence among conspecific populations. Although many population genetic surveys of marine species with highly dispersive stages have found little evidence to support the hypothesis that present day current patterns influence genetic divergence among contemporary marine populations (e.g. Benzie and Williams 1995, Shulman and Bermingham 1995), other studies such as those of Tracey et al. (1975) and Rocha-Olivares and Vetter (1999) observed that genetic structure for lobsters and rockfish, respectively, coincided with regional oceanic circulation patterns. In the Gulf of Maine, the Eastern Maine Coastal Current (EMCC) is a prominent oceanographic feature and is known to transport herring larvae from spawning grounds near Grand Manan Island westward along the coast of Maine (Townsend 1992). The EMCC also appears to have a significant influence on the distribution of *Alexandrium* sp., a dinoflagellate responsible for paralytic shellfish poisoning. The lowest incidence of paralytic shellfish poisoning along the coast of Maine occurs between the western edge of Penobscot Bay and Jonesport, ME (Shumway et al. 1988). In this region, *Alexandrium* is found pre-

dominantly offshore and its distribution coincides with an offshore-directed tongue of cold water near Jonesport (Dave Townsend, pers. comm.). The dispersive larval stage in blue mussels typically spans upwards of at least 3 to 4 weeks (Bayne 1976). Given a mean current velocity in the EMCC approaching 15–25 cm s^{-1} (Townsend 1992) there is the potential for any *M. trossulus* larvae entrained in the EMCC to be transported great distances to the west. Moreover, the offshore flow of the EMCC near Jonesport, ME is particularly evident in satellite imagery from the summer months (Fig. 4; see also Pettigrew et al. 1998) when the bulk of mussel larvae are in the water column along the coast of Maine. Thus, it is possible that any *M. trossulus* larvae transported west further into the Gulf of Maine may not be retained in nearshore waters at a time when they are competent to settle, and the relative strength and direction of the EMCC then, determines the extent to which the range of *M. trossulus* extends into the Gulf.

Temperature variation has also been investigated as a potential mechanism structuring the species composition of blue mussel populations in the northwest Atlantic (Mallet and Carver 1995). Given the broad-ranging distribution of *M. edulis*, the more restricted sub-polar distribution of *M. trossulus*, and the absence of *M. trossulus* from the Northumberland Straits between Prince Edward Island and New Brunswick, Canada, it has been suggested that the latter species is less tolerant of warmer waters (e.g. Gosling 1992b, Mallet & Carver 1995). Mallet and Carver (1995) examined the relative survival and growth of *M. trossulus* and *M. edulis* at two locations differing in mean temperature in Nova Scotia. Their findings, that *M. trossulus* demonstrated higher survival rates at the warmer site, were the reverse of what would be predicted based on the large scale distribution of *M. edulis* and *M. trossulus*. Their study, however, was limited to a comparison of the survival of adult mussels in two size classes (20–30 and 40–55 mm shell length).

In contrast, Bayne (1976) has argued that the environmental requirements for normal embryonic development are likely to be a major factor limiting the distribution of blue mussel populations. If so, then attempts to correlate adult distributions with spatial variation in environmental parameters are likely to be of limited value in determining the mechanisms regulating the species composition of mussel populations in the Gulf of Maine. More attention should be focused on processes affecting the early life history stages of mussels. In this regard, satellite imagery suggests that there may be a significant change in coastal water temperature along the coast of Maine, coincident with the offshore movement of the EMCC. East of Jonesport, where the EMCC remains close to shore, the nearshore waters remain relatively cool throughout the summer compared to those west of Jonesport. Thus, the EMCC may impact blue mussel larvae in two ways, in a direct manner wherein *M. trossulus* larvae are advected away from potential settlement sites and an indirect manner wherein the EMCC creates a temperature differential between eastern and western Maine that affects the survival of *M. trossulus* larvae. We have recently initiated a series of experiments designed to assess the relative importance of these two mechanisms in limiting the range of *M. trossulus* in Maine.

In conclusion, our results clearly show that *M. trossulus* occurs, and in some cases is the predominant species in mussel populations in eastern Maine. This study sampled two locations, Machiasport and Gove Point, frequently sampled by the Gulfwatch program (Hennigar et al. 1996). The marked change in species composition between these two sites illustrates the importance of monitoring the frequency of *M. trossulus* at Gulfwatch sites in the northeastern Gulf of Maine to facilitate the interpretation of Gulfwatch data. The findings of Mallet and Carver (1995) regarding the economic impact of *M. trossulus* on mussel aquaculture also highlight the importance of monitoring species composition at current and future mussel culture lease sites to enable the industry to select sites that limit the presence of this species. Additional work is now underway in our laboratory. We are attempting to better define the factors that control the distribution of *M. trossulus* throughout the Gulf of Maine, to further understand how the species composition of Gulf of Maine populations may change in the future.

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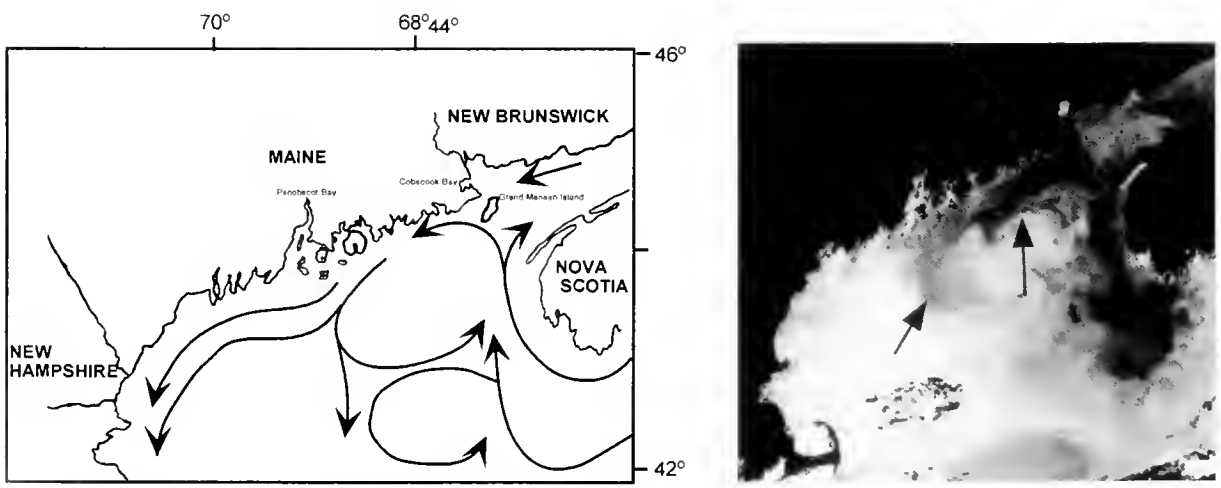

Figure 4. The map on the left provides a diagrammatic representation of the general circulation patterns (arrows) in the Gulf of Maine during stratified conditions typical in this region from May to September (after Pettigrew, unpublished). The figure on the right is an AVHRR satellite image of sea surface temperature in the Gulf of Maine on July 19, 1998. Cooler water appears as darker shades and warmer water as lighter shades in this image. The eastern Maine coastal current and an offshore-directed plume are indicated by the arrows. Image courtesy of the University of Maine Satellite Oceanography Data Laboratory.

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THE EFFECT OF TEMPERATURE ON THE REPRODUCTIVE MATURITY OF THE PENSHELL *ATRINA MAURA* (SOWERBY, 1835) (BIVALVIA: PINNIDAE)

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ABSTRACT The effect of temperature on reproductive maturity of recently spawned penshell broodstock *Atrina maura* (Sowerby 1835) was studied. Penshells were maintained in an open-flow system and were fed to satiation with a mixture of microalgae. Condition index (tissue wet weight/total wet weight × 100), the rate of maturation, and oocyte quality were followed histologically until spawning. A scheme of six stages, based on histological preparations, was found best to describe the maturation process in the penshell. Results indicate that penshell maturation must be at low temperatures over two months to obtain high quality oocytes in the hatchery, even though the maturation period can be shortened to half that time at higher temperatures. A reduction of the quality of oocytes is obtained at higher temperatures.

KEY WORDS: reproductive maturity, temperature, condition index, penshell, atresia, *Atrina maura*

INTRODUCTION

Along the Pacific coast of Mexico, five species of penshell bivalves occur (Keen 1971). *Atrina maura* (Sowerby 1835) is the species with the highest value adductor muscle. Over the past ten years, exploitation of penshell natural stocks has led to a decline in production (Velez-Barajas & Fajardo-León 1996, Cardoza-Velasco & Maeda-Martinez 1997). Penshells are vulnerable to overexploitation because they have a long life, limited reproductive investment, and sporadic recruitment (Butler et al. 1993). Because of the decline in production, an increased interest in the cultivation of *A. maura* was stimulated (Baqueiro & Castagna 1988, Arizpe-Covarrubias 1995, Reynoso-Granados et al. 1996). Information on the biology and aquaculture of *A. maura* is still scarce. In this species, tolerance to temperature and salinity and the optimum temperature for growth (Leyva-Valencia 1999), as well as the gonadic cycle in Bahia Magdalena (Maeda-Martínez unpublished data) and the growout method in the field (Cardoza-Velasco & Maeda-Martinez 1997) was determined. Two Mexican institutes (Centro de Reproducción de Especies Marinas in Sonora (CREMES) and at CIBNOR) produced a limited number of spat used mainly for scientific purposes. However, there are no studies on hatchery reproductive maturation of this species.

In other members of the family Pinnidae, such as *Pinna rugosa*, reproductive biology (Noguera & Gómez-Aguirre 1972, Coronel 1981) and recruitment (Arizpe & Félix 1986, Arizpe-Covarrubias 1987, Arizpe-Covarrubias 1995) was studied. Oogenesis and spermatogenesis have been described at an ultrastructural level in *Pinna nobilis* (de Gaulejac et al. 1995a, b), as having the ultrastructural changes of maturing oocytes in *Atrina pectinata* (Yongqiang & Xiang 1988).

Endogenous and exogenous factors control both gametogenesis and spawning (Barber & Blake 1991). Among the exogenous factors, the most important are temperature and food (Sastry 1963, Loosanoff & Davis 1963, Sastry 1966, 1968, Giese & Pearse 1974, Lowe et al. 1982, Taylor & Capuzzo 1983, Barber & Blake 1991). Mollusks can be caused to mature artificially out of season by increasing temperature and food (Loosanoff & Davis 1963). A threshold temperature is needed for vitellogenesis to continue and effects the transfer of nutrients necessary for the growth of oocytes (Sastry 1968, 1970, Sastry & Blake 1971, Blake 1972, Sastry 1979). The quality of oocytes can be assessed by the size of the oocytes at the postvitellogenic stage, and by examining oocyte morphology. The mean oocyte dimensions observed in histological sections is a reflection of the gametogenic cycle, because oocytes gradually increase in size as they develop, reaching maximum size prior to spawning (Barber & Blake 1991). However maximum size can vary depending on oocyte nutrition during vitellogenesis and therefore this size has to be determined experimentally under different exogenous conditions. Oocyte morphology is another criteria used to determine its quality. A common phenomenon usually observed in mollusks is oocyte degeneration (atresia) at the end of vitellogenesis because of the presence of lytic enzymes that spread throughout the acinus (Beninger & Le Pennec 1991).

To find the optimum temperature for reproductive maturation of recently spawned *A. maura*, we determined both the condition index and the rate of maturation and oocyte quality at different temperatures. As a basic reference, a histological study was done to describe the phases of reproductive maturation in this species.

MATERIALS AND METHODS

About 100 penshell (*A. maura*) specimens (98 ± 0.1 mm shell length) were brought to the laboratory at CIBNOR from the aquaculture farm Rancho Bueno, located at the southernmost end of Bahía Magdalena on the Pacific coast of Baja, 137 km northwest of La Paz. These animals were produced originally at CREMES and were then sent to Rancho Bueno for growout in Nestier trays suspended from a 100-m longline. By the time of collection on July 15, 1995, the animals were six months old and had fully developed gonads. Specimen spawning was easily induced in the laboratory by using a thermal shock. After spawning, the penshells were washed with seawater and divided randomly into three groups. They were then placed in three 1,500-L fiberglass tanks at 20, 25, and 30°C, vertically in plastic racks to simulate their natu-

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ral position in the field. To eliminate an effect of food shortage on gonad maturation, microalgae was supplied continuously during the experiments. Each tank received a constant flow of 83-L h^{-1} of seawater at 37‰ salinity, containing a mixture of microalgae (*Isochrysis galbana*, *Monochrysis lutheri*, and *Chaetoceros gracilis*) at a concentration of 130–155 cells μL^{-1}. At this flow rate, the tanks received a daily water exchange of 133%. Counts of microalgae at the outlet ports of the tanks varied between 15 and 60 cells μL^{-1} depending on the number of animals held in the tanks throughout the experiments. The quality of these microalgae species on bivalve nutrition is well documented (Walne 1970). The water used in the experiments was drained from a 200-L tank where the cultured algae were mixed with flowing filtered seawater. The algae were kept well mixed in suspension by using an air bleed at the bottom of the tank. The mixture was monitored automatically with a Hach turbidimeter Model 1720C to make certain the algal concentration was constant. The turbidimeter operated a 1/32-hp submersible pump that transferred the microalgae to the mixing tank. The turbidimeter was set to turn on the pump when turbidity dropped below a preset value equivalent to the desired algal suspension of a standard calibration curve. The temperature in the 20°C tank was maintained with a 1/8 hp Acrytech water chiller. The temperature in the 25°C tank was held by setting the air conditioning system of the laboratory to that temperature, and the temperature in the 30°C tank was achieved by using six 200-W thermostatically controlled immersion heaters. The tanks were cleaned every day, removing feces with a plastic siphon hose.

To determine the influence of temperature on gonad maturation, six penshells were analyzed histologically at the start of the experiment and an equal number of animals from each tank was taken, and examined every 15 days (15, 30, 45, and 60 days). Tissues were fixed in Davidson's solution, dehydrated with an increasing concentration of ethanol (70% to 100%), and embedded in Paraplast at 56°C. Thin sections (6 μm) from the gonad area were cut with a rotary microtome and stained with hematoxylin and eosin (Howard & Smith 1983). From these sections, a schedule of the phases of gonad maturation in the penshell was termed. The quality of postvitellogenic oocytes at the different temperatures was evaluated by determining the nucleus-cytoplasm ratio and the presence or absence of atresia. The nucleus and cytoplasm of oocytes were measured with the Sigma Scan image analyzer software on images taken with a Zeiss light microscope Model 16. Only oocytes sectioned through the nucleolus were measured. Atresic oocytes were considered those in which the basophilic properties of the nucleus was lost, and the oocytes took a "jigsaw-puzzle" appearance (Barber and Blake 1991). Care was taken to record any spontaneous spawning in the tanks and also to fix for histological analyses the tissues of any specimen at the moment of spawning. The histological sections were used to define the different stages of gonad development in this species, taking into consideration morphology, presence of particular structures, and degree of vitellogenesis. Before cutting and embedding the tissues, a condition index was estimated by dividing the tissue wet weight of recently dissected animals by total wet weight, multiplied by 100.

RESULTS

Microscopic observations of gonadal sections from the different temperature treatments allowed us to determine that *A. maura* is a dioecious species with two previtellogenic (Stages I and VI), two vitellogenic (Stages II and III), one postvitellogenic (Stage IV), and one spent stage (Stage V) in the female gonad. Six stages were found that clearly describe spermatogenesis in the male gonad. We observed no hermaphroditism.

Female Gonad Maturation Stages

Stage I. Early Active

The acini are irregular in shape and are supported by interfollicular connective tissue (Fig. 1a). The acini stem cells give rise to primary oogonia 3–5 μm in diameter. The nucleus is round and contains a round nucleolus. In this stage, previtellogenic oocytes are round with a maximum diameter of 25 μm, and are attached to the acini.

Stage II. Developing

Acini walls are well defined and the interfollicular connective tissue has decreased (Fig. 1b). Acini acquired a circular shape with oocytes attached to the periphery. Oocytes enter the vitellogenic phase, which is evidenced by a rapid growth of the cytoplasm (lengthwise), reaching up to 60 μm in length. The nucleus also attains an elongated shape. It has a single nucleolus (5 μm) located centrally or marginally within the nucleoplasm. Oocytes are pedunculated and show dense aggregates in the stalk region, arranged in narrow rods perpendicular to the acini walls (Fig. 1c). These aggregates may correspond to the clusters of mitochondria arranged in line alongside cytoplasmic microtubules described by de Gaulejac et al. (1995a) in *Pinna nobilis*. Some elongated cells appear in the stalk region and are in close contact with the oocytes (Fig. 1d). These may play a role as auxiliary cells in the transfer of nutrients to the oocytes (de Gaulejac et al.1995a).

Stage III. Late Active

Oocytes continue to grow and attain a polyhedral shape (Fig. 1e) ranging from 45–57 μm in diameter. Some oocytes appear free in the lumen and others remain attached to the acini walls. The nucleus is round and occupies a large area in the middle of the oocyte. A dense amorphous mass (probably chromatin) is frequently observed in the nucleoplasm. The nucleolus decreases in size to 3.3 μm diameter, and appears surrounded by a nucleolar ring (Fig. 1e). From this stage on, a large and conspicuous dense aggregate (Fig. 1e) appears outside the nucleus, which may correspond to the 7–8 layered Golgi complex observed by Yongqiang and Xiang (1988) in *Atrina pectinata*. Auxiliary cells are no longer observed.

Stage IV. Mature

This stage marks the end of the vitellogenic process. Postvitellogenic oocytes measure a maximum of 56 μm along the major axis, and maintain their polyhedral shape because of oocyte crowding (Fig. 2a). Interfollicular and interoocyte spaces are minimal. Oocytes appear free in the lumen of the acinus. The dense aggregate still appears outside the nucleolar envelope.

Stage V. Spawning

Acini appear full of mature postvitellogenic oocytes with a broken germinal vesicle (Fig. 2 b). The dense aggregate and the nucleoplasm with the nucleolus appear spread along the ooplasm. The polyhedral shape is maintained because of oocyte crowding in the acini.

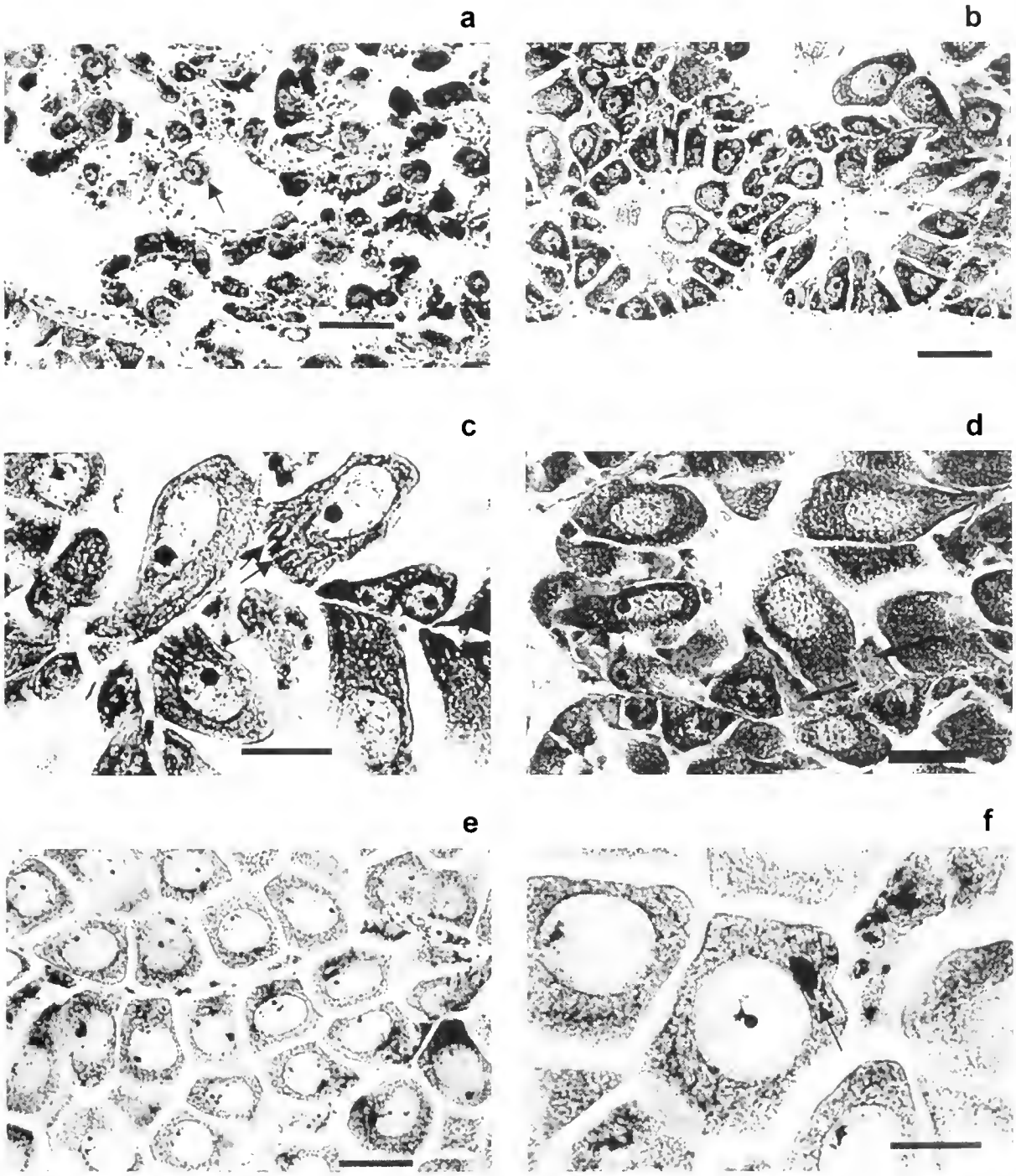

Figure 1. Light micrographs of stages of reproductive maturity in female *Atrina maura*, at 20° C. (a) Early active (stage I). Arrow shows an oocyte under formation within the acinus, bar = 50 µm. (b) Developing (stage II). Two acini with oocytes growing toward the lumen, bar = 50 µm. (c) Oocytes form stage II, showing clusters of mitochondria at the stalk, in line with microtubules (arrows), bar = 25 µm. (d) Oocyte from stage II with auxiliary cells at the stalk region, bar = 25 µm. (e) Oocytes at late active stage (stage III), bar = 50 µm. (f) Dense aggregate (arrow) on the external wall of nuclear envelope of a stage III oocyte, bar = 25 µm.

Stage VI. Spent

The acini are collapsed as a result of oocyte evacuation (Fig. 2c). A few unspent oocytes with the germinal vesicle intact remain in the acini in the process of degradation. Hemocytes proliferate during this stage, contributing to gonad repair.

Male Gonad Maturation Stages

Stage I. Early active

Acini look elongated (Fig. 3a). The germinal epithelium produces spherical spermatogonia 3-µm diameter after a centripetal

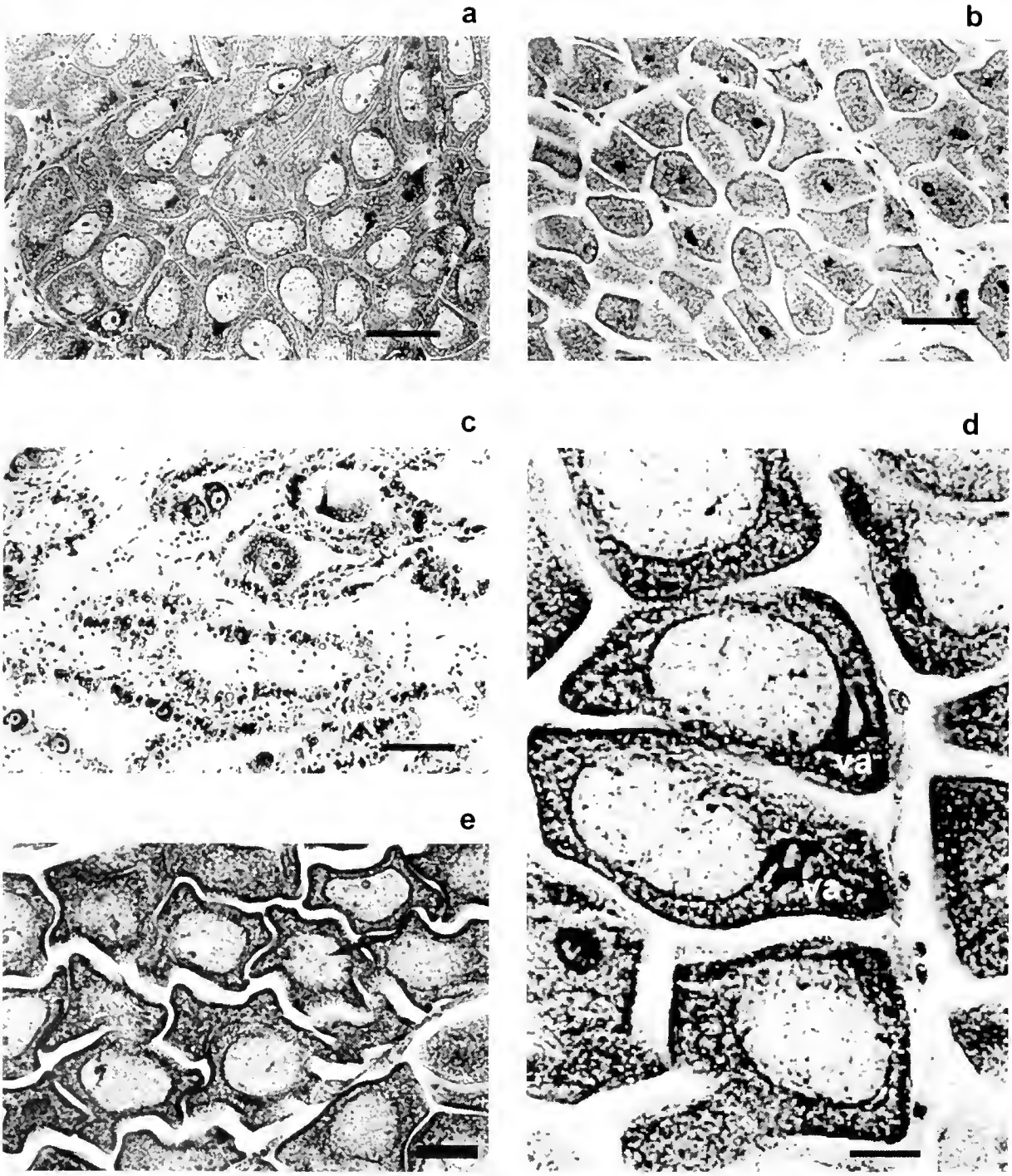

Figure 2. Light micrographs of stages of reproductive maturity in female *Atrina maura*. (a) Polyhedral oocytes at mature stage (stage IV) at 20°C, bar = 50 µm. (b) Oocytes at spawning stage (stage V) with broken germinal vesicles at 20°C, bar = 50 µm. (c) Spent gonad (stage VI) with residual non-spawned oocytes at 20°C, bar = 50 µm. (d) Overmaturated (atresic) oocytes (stage IV) showing vacuoles probably resulting from distension of endoplasmic reticulum at 30°C, bar = 25 µm. (e) Gonad with "jigsaw-puzzle" shaped overmaturated (atresic) oocytes (stage IV) at 30°C. Nuclei of some oocytes have lost their basophilic properties (arrows), bar = 20 µm.

evolution from the internal wall to the lumen. Spermatogonia are located along the internal wall of the acini in bands of several cells. Some spermatocytes are present in the lumen.

Stage II. Developing

Acini begin to show stratification (Fig. 3b), and all developmental stages are present including spermatogonia, abundant spermatocytes in the following layer, spermatids, and scarce spermatozoa located toward the acinus lumen. Cell diameter decreases from 3 to 0.5 µm.

Stage III. Late Active

Spermatogenesis takes place in the whole area of acini (Fig. 3c). The spermatogonia layer becomes thinner. The number of spermatozoa increase and their tails are directed toward the lumen.

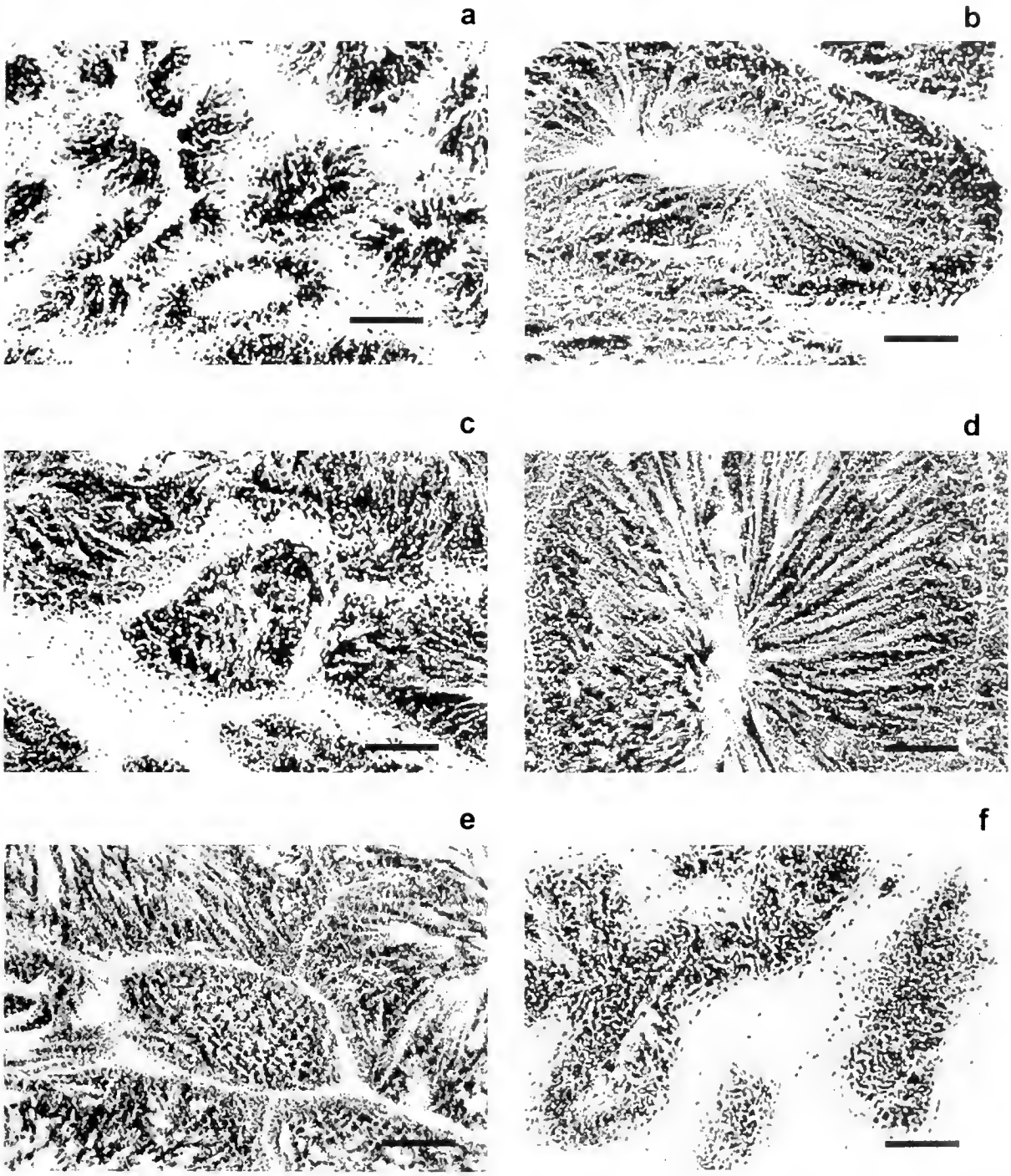

Figure 3. Light micrographs of stages of reproductive maturity in male *Atrina maura*, from 20 C treatment. (a) Early active (stage I) showing spermatoogonia at the periphery of acini, bar = 50 µm. (b) Developing (stage II), bar = 50 µm. (c) Late active (stage III), bar = 50 µm. (d) Mature (stage IV), bar = 50 µm. (e) Spawning (stage V), bar = 50 µm. (f) Spent gonad (stage VI) partially spawned, bar = 50 µm.

Stage IV. Mature

Acini look full of spermatozoa, with their tails toward the acini lumen (Fig. 3d). Merging of some acini is evident.

Stage V. Spawning

Acini become elongated and the boundaries between acini are not easily distinguished (Fig. 3e). Evacuating ducts are evident from which a great number of spermatozoa are spent.

Stage VI. Spent

Numerous empty spaces toward acini lumen are evident as a result of the release of spermatozoa during spawning (Fig. 3f). As in the female gonad, spawning is not complete. Some acini are empty but others remain full of ripe sperm. The empty acini show a great number of hemocytes. No signs of active spermatogenesis are observed in any region of the gonad.

Effect of Temperature on Maturation

The results of the histological analyses at different temperatures and sampling dates indicate synchronous gonad maturation in male and female gonads. In addition, a faster gonad maturation rate was observed at 25 and 30°C than at 20°C (Fig. 4). At the beginning of the experiments, more than 80% of the organisms showed immature gonads (stages I and VI) as a product of spawning induced by thermal shock. Only 20% were in stage II, indicating that gametogenesis can occur rapidly just after spawning. At day 15, samples from 20°C were only at stages I and II. At 25°C, 70% of the organisms were also in stage II, but the rest had reached developing stage III. At 30°C, the majority of the organisms (70%) were in stage III and the remaining 30% had reached maturity (stage IV). By day 30, a small proportion of animals (18%) at 20°C was still in stage II, but the rest had advanced to stage III. All specimens analyzed at 25°C had advanced to stage IV, but maturation rate in 30°C organisms did not show any progress. After this sampling date, spontaneous spawning was recorded at 25°C on day 34 and at 30°C on day 41. The results from spawning were only confirmed histologically on day 45 at 25°C, where 60% showed spent gonads. The remaining 40% had not spawned, showing gonads still in stage IV. Histological confirmation was not obtained at 30°C by day 45. In this treatment and date, relative frequencies remained the same as in days 15 and 30, with 70% and 30% in stages III and IV. Relative frequencies of penshells at 20°C on day 45 were 50% and 50% in stages II and IV. At 20°C, nearly 80% of the organisms were in stage III and the rest in stage IV on day 60, and spawning of these organisms was also recorded on day 60.

Oocyte Size and Nucleus-Cytoplasm Ratio

Results from postvitellogenic oocyte measurements at different temperatures are shown in Table 1. From this, oocytes at 20°C show on average a larger area (1660 μm^2) and major axis (56 μm) than those at 25°C (1416 μm^2 and 53 μm) and 30°C (1290 μm^2 and 49 μm). ANOVA indicates a significant difference between oocyte sizes at different temperatures at $\alpha = 0.05$. A Tukey's multiple range test showed that oocyte areas at 25 and 30°C were similar at $\alpha = 0.05$, but were significantly different from those at 20°C.

The areas of the nucleus varied between 578 and 564 μm^2 at different temperatures, but the ANOVA identified no significant differences between temperatures. With these data, we calculated a nucleus-cytoplasm ratio that was lower (0.34) at 20°C than at 30°C (0.43). This indicates a reduction in cytoplasm area at higher maturation temperatures.

Atresia

A second way to demonstrate oocyte quality related to maturation temperature was the presence of atresic oocytes. Results from histological observations indicate that atresic oocytes were not present in animals held at 20°C but were observed in stage III and IV female gonads at 25°C and 30°C (Fig. 2e). In this Figure, one can see the nucleus of atresic oocytes has lost its basophilic properties, and the oocytes have a "jigsaw-puzzle" appearance. Atresic oocytes were smaller than normal oocytes. The cytoplasm of nonatresic oocytes at 25 and 30°C showed large vacuoles (Fig. 2f) at the dense aggregate outside the nucleus. Vacuolation of this kind was reported in atresic oocytes of *Pinna nobilis* (de Gaulejac et al. 1995a), as a result of distension of endoplasmic reticulum.

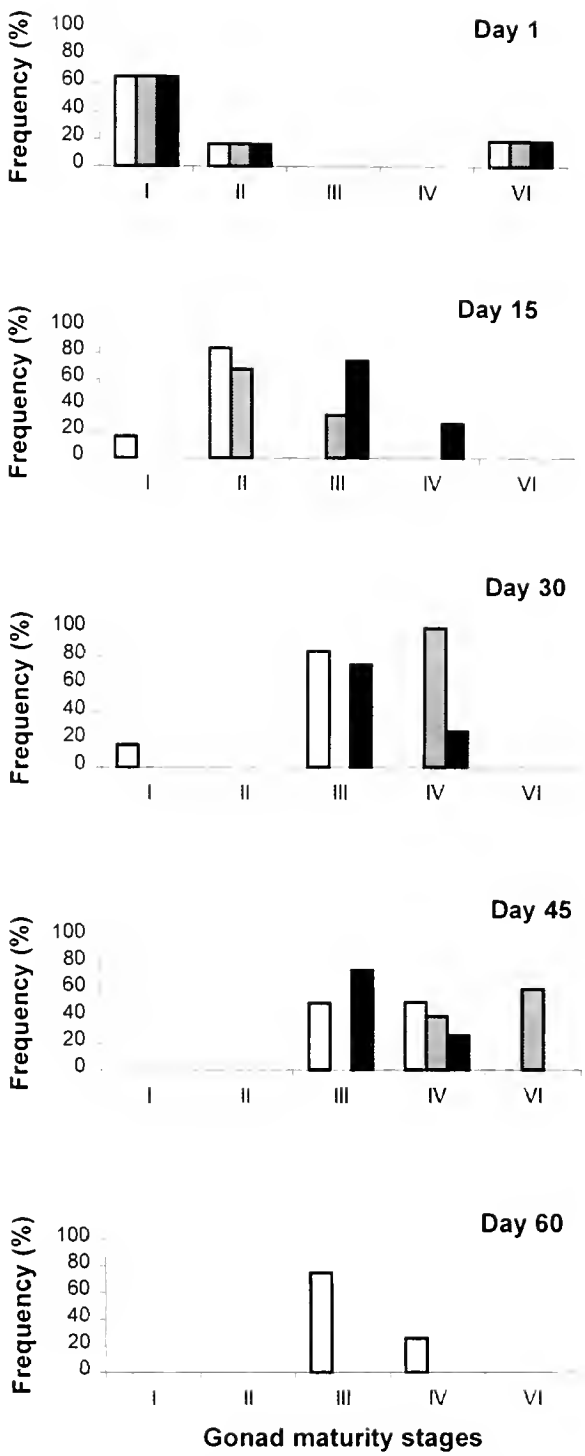

Figure 4. Relative frequency of stages of reproductive maturity in *Atrina maura*, at different maturation times and temperatures (white columns = 20°C, gray columns = 25°C, black columns = 30°C).

Condition Index

Condition index (CI) at 20°C increased steadily from 32% at the beginning of the experiment to 52% at day 60 when spawning occurred (Fig. 5). However, at 25°C and 30°C, only small increases were measured during the maturation period. At these temperatures, CI increased rapidly from 32% to 38% in the first 15

TABLE 1.

Dimensions of postvitelogenic oocytes and nucleus of penshell (*Atrina maura*) and the nucleus-cytoplasm ratio (N:C), at different temperatures.

Temperature (°C)	Oocyte			Nucleus			N:C Ratio
	Area (μm^2)	Major Axis (μm)	Minor Axis (μm)	Area (μm^2)	Major Axis (μm)	Minor Axis (μm)	
20	1660 ± 300	56 ± 6	42 ± 6	578 ± 101	31 ± 3	25 ± 3	0.34
25	1415 ± 182	53 ± 4	39 ± 5	564 ± 83	31 ± 3	24 ± 3	0.39
30	1290 ± 234	49 ± 6	37 ± 5	564 ± 110	30 ± 3	24 ± 3	0.43

Figures are the mean ± standard deviation (n = 90).

days, but no further increases were observed from day 15 to day 30 at both temperatures. From days 30 to 45, minor increases of 1% and 8% were recorded at 25°C and 30°C. Spawning at these temperatures occurred at days 34 and 41. No effect of spawning on CI was detected at these temperatures.

DISCUSSION

In the literature, many schemes for classifying gonad condition in mollusks can be found (Chipperfield 1953, Lubet 1957, Sastry 1963, Wilson & Hodgkin 1967, Lunetta 1969, Seed 1969, Villalejo-Fuerte & Ochoa-Báez 1993, de Gaulejac et al. 1995a, de Gaulejac et al. 1995b, Villalejo-Fuerte et al. 1995). We developed a specific scheme for *Atrina maura* based on morphological observations of gametes and specific structures. The scheme developed included six stages that clearly describe the gonad maturation process in *A. maura*. Although our work was conducted using light microscopy, interpretation of several structures was possible using the ultrastructural descriptions of gonad maturation made by Yongqiang and Xiang (1988) and de Gaulejac et al. (1995a,b), for the pinnids *Atrina pectinata* and *Pinna bicolor*. This study revealed maturation of male and female gonads occurs synchronously, and progressed evenly among the animals in the same treatment.

Morphological observations allowed us to compare the oocytes of *Atrina maura* with those described in *A. pectinata* and *Pinna nobilis*. Results indicate that the oocytes of *Atrina* species are morphologically similar, but three interesting differences exist with the oocytes of *P. nobilis*. The margin of the nucleus of *P. nobilis* is polylobed and twisted, whereas in *Atrina* the nuclear envelope is even and lacks indentations throughout development. The nucleolus in *P. nobilis* is located at the edge of the nucleus, whereas in *Atrina* it can either be at the edge or in the center. Finally, the dense aggregate (presumably a Golgi complex) found outside the nucleus of *Atrina* oocytes is not conspicuous in *P. nobilis*.

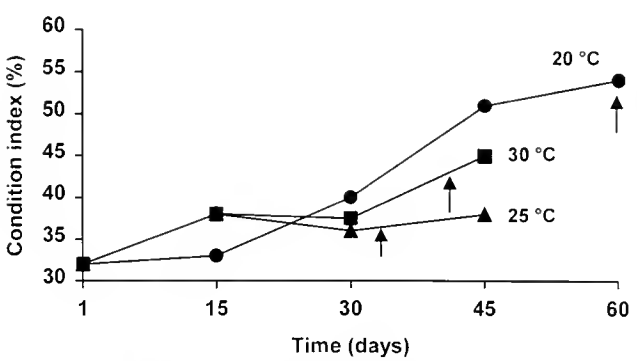

Figure 5. Changes in condition index of *Atrina maura* at different temperatures. Arrows indicate the day of spontaneous spawning.

Previous studies on the reproductive biology of bivalve mollusks have demonstrated that temperature and food are the most important factors influencing the reproductive cycle of marine invertebrates (Loosanoff & Davis 1963, Sastry 1963, Giese & Pearse 1974, Lowe et al. 1982). We found an effect of temperature on the rate of gonad maturation and the quality of oocytes in *A. maura*. The effect of food on these factors was eliminated by feeding the organisms to satiation with high quality species of microalgae in all treatments. Differences in the rates of gonad maturation and quality of oocytes are therefore attributed exclusively to temperature. The gonad maturation period was shorter at 25°C and 30°C than at 20°C, possibly because of the higher metabolic rates found at higher temperatures in this species. The scope for activity and shell growth rate measurements indicate the range of optimum temperature for growth in juvenile *A. maura* was 25–29°C (Leyva-Valencia 1999, Leyva-Valencia et al. 2001). With this knowledge, and desiring the shortest conditioning period possible, we suggest that penshell gonad maturation should be done at a temperature above 25°C. However, the results from histological observations on the quality of oocytes may lead to a different conclusion. Larger and nonatresic oocytes were only found at 20°C, probably because of a longer vitellogenic phase that allowed the incorporation of larger amounts of nutritive materials. Spawning from unfed or stressed broodstock results in weak batches of larvae that fail to undergo normal pelagic development (Loosanoff & Davis 1963, Bayne et al. 1984). The correlation between both factors on the fecundity and larval viability in *A. maura* needs to be determined. Coincidentally, spawning of *A. maura* in Bahía Magdalena takes place during the coldest season of the year (from January to March) when water temperature varies between 19.5 and 20°C (Maeda-Martínez unpublished data).

We measured condition index (CI) based on tissue and total wet weights of the animals. This is not a refined method but it proved to be a good alternative for measuring CI in *A. maura*. One of the problems that limited the use of a more precise method was the gonad in this species is included in a visceral mass together with other tissues, and it is not possible to obtain its weight excluding other tissues. Clear differences were observed between CI from the different temperature treatments and these correlated positively with oocyte size. Therefore, CI reflected variation in gonad size assuming that fecundity was the same among treatments. This means that smaller ("immature") oocytes were spontaneously spawned at 25°C and 30°C. The lower cytoplasmic content in

these oocytes was confirmed with the results from the nucleus-cytoplasm ratio. The relation between oocyte size and viability of resulting penshell larvae and spat remains to be tested.

Another factor that may have contributed to the depletion of CI at higher temperatures (25–30°C) was the formation of atresia. CI depletion was observed after the first 15 days from the start of the experiments (Fig. 5) at the beginning of vitellogenesis when atresia normally occurs (Barber & Blake 1991). The acceleration of maturation at high temperatures (25 and 30 C) resulted in necrotic oocytes, probably by the secretion of lytic enzymes (Beninger & Le Pennec 1991). Prolonged exposure of *Argopecten irradians* to subthreshold temperature, with oocytes undergoing vitellogenesis, results in vacuolization of the cytoplasm as we observed in *A. maura* (Fig. 2d) and lysis of oocytes (Sastry 1966, 1968). Although oocyte degeneration is a commonly observed phenomenon in species whose gametogenesis is under the control of natural environmental conditions (Pipe 1987), further studies are needed to find the correlation between temperature and secretion of lytic enzymes. Temperature did not affect the morphology of male gonads. Again, the viability of sperm released from animals matured at different temperatures remains to be tested.

In conclusion, results from the present work indicate that to obtain high-quality oocytes in the hatchery, penshell maturation must be done at low temperatures (20°C) over two months. Even though the maturation period could be shortened to half this time at higher temperatures, the quality of oocytes is greatly reduced.

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HALOTOLERANCE, UPPER THERMOTOLERANCE, AND OPTIMUM TEMPERATURE FOR GROWTH OF THE PENSHELL *ATRINA MAURA* (SOWERBY, 1835) (BIVALVIA: PINNIDAE)

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ABSTRACT Halotolerance, upper thermotolerance, and optimum temperature for growth was investigated in juvenile penshell (*Atrina maura*). Halotolerance and thermotolerance were determined measuring LD_{50}-96h within the salinity and temperature ranges of 15–50‰ and 19–35°C. Optimum temperature for growth was measured estimating scope for activity, ingestion, and clearance rates at 16, 19, 25, and 29°C. Growth rates were also measured over 42 days at the same temperatures. Results indicate that salinity LD_{50}-96h ranged from 16‰ to more than 50‰, and the upper temperature LD_{50}-96h was 33.2°C. Scope for activity, ingestion, clearance, and growth rates were highest at 29°C indicating that optimum temperature in penshell juveniles is 29°C or higher. Because of the wide halotolerance range and the high thermotolerance and optimum temperature, the penshell is a good candidate for aquaculture in subtropical and tropical areas.

KEY WORDS: *Atrina maura*, penshell, salinity tolerance, temperature tolerance, temperature optimum

INTRODUCTION

The penshell *Atrina maura* Sowerby, 1835, is a bivalve mollusk that lives in shallow coastal lagoons and estuaries along the Pacific coast from Baja California to Peru (Keen 1971). It has a large adductor muscle of great value in Mexican and international markets. Natural populations in Mexico have decreased because of overfishing (Reynoso-Granados et al. 1996). Because of this, there is an increasing interest to develop aquacultural techniques for this species. Aquacultural methods are under development (Maeda-Martínez et al. 1996). It appears that a combination of suspension culture for the nursery stage and bottom culture for growout will be adequate for the species (Reynoso-Granados et al. 1996, Maeda-Martinez, A.N. unpublished results). Growout in bottom culture lasts two years (Cardoza-Velasco 1998), allowing then harvesting of meats of 14–26 g fresh weight (Cardoza-Velasco unpublished results) at 9–11USD/kg in the Mexican market (Velez-Barajas and Fajardo-León 1996, Cardoza-Velasco 1998).

The tolerance limits to salinity and temperature and the optimum temperature for growth have not yet been determined. For *A. maura*, knowledge of the limits of these variables are important to optimize hatchery production and to select sites for aquaculture in the field. Tolerance limits have been determined in other mollusks (Al-Habbib & Graingner 1977, Wilson 1978, Ansell et al. 1980, Ansell et al. 1981, Poza-Boveda & Rodriguez 1987, Quinn et al. 1994, Sicard-González 1999).

Physiological methods such as scope for activity and clearance and ingestion rates are good tools to determine optimum values, as these correlate well with actual growth measurements (Sicard et al. 1999). Scope for activity has been used as a good indicator of the energy available to the organism to do other functions, apart from basic maintenance, such as growth, reproduction, movement, and regulation against variations in external factors (Fry 1947). Scope for activity is the arithmetic difference between active and standard respiration rates. The former is obtained from an individual fed to satiation and the latter from an organism kept under long-term starvation. Under starvation, respiration rate gradually drops to a steady-state in several days. Scope for activity is then determined over a wide range of temperatures, and the optimum value would be where the difference between active and standard respiration rates is a maximum.

Other physiological factors such as clearance and ingestion rates have been found in the catarina scallop to correlate with scope for activity over a wide range of temperatures (Kirby-Smith 1970, Lu & Blake 1997, Sicard et al. 1999). This needs to be confirmed because optimum values for growth could be determined in the future by making only clearance rate experiments.

In the present work, tolerance limits to salinity, the upper tolerance limit of temperature, and the optimum temperature for growth of penshell juveniles have been studied.

MATERIALS AND METHODS

Experimental Animals

Penshell (*A.maura*) juveniles were produced in the hatchery at CIBNOR following a technique developed by Robles-Mungaray (unpublished results). These were approximately 5-months old when used, and measured 23 ± 10 mm shell height. To standardize the results, the relationship between shell height and dry tissue weight of 24 juveniles of the size range used in the experiments was calculated. Shell height was found to be a better characteristic than shell length, because the shell margin could be easily damaged during measurements. To obtain the dry weight of the tissues, the whole animals were dried in an oven at 65°C for 36 h and then the tissues removed from the valves with a dissection needle. The tissues were weighed on an electronic OHAUS microbalance, model Galaxy 110 with 0.1 mg resolution.

Halotolerance and Upper Thermotolerance

Halotolerance of penshell juveniles was determined following the standard procedure of LD_{50}-96 described by Rand and Petrocelli (1985). This consists of recording survival at predetermined times up to 96 h in animals exposed to a range of a given variable, i.e. temperature, salinity, toxic substance. Before the experiments, the juveniles were maintained for 14 days in 70-L plastic tanks containing 40 L of seawater at 22°C and 38‰ salinity. During this acclimation period, the tanks received constant aeration and the animals were fed 4×10^9 cells/penshell/day of a 1:1 mixture of the

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microalgae, *Isochrysis galbana* and *Chaetoceros gracilis*. Fifty percent of the water was changed every day.

At day 14, groups of seven juveniles, in triplicate, were transferred from the acclimation tanks to 3.5-L containers with seawater at 22°C and at 15, 20, 25, 30, 38, 42, 45, and 50‰ salinity. The water for salinity treatments below 38‰ was prepared by diluting seawater with distilled water, and those at higher salinity with sea salts added to give the desired salinity. During the experiments, 50% of the water was changed daily and the animals were fed with the same mixture of microalgae. Survival was recorded at 4, 8, 72, and 96 h.

Upper thermotolerance was determined in penshell juveniles following the same method for halotolerance. The range investigated was 19°C to 35°C. Groups of seven juveniles in triplicate were transferred to the 3.5-L containers with water at 38‰, and at 19, 22, 24, 28, 30, 32, and 35°C. The 19°C treatment, which was colder than the temperature in the laboratory (22°C), was achieved by placing the containers in a refrigerated waterbath, and in the rest of the treatments, a 200-Watt thermostatically-controlled heater was introduced to each container. The water was constantly aerated to assure good mixing. Fifty percent of the water was changed daily and the animals were fed with the same mixture of microalgae used in the previous experiments. Survival was recorded at 4, 8, 72, and 96 hours.

Optimum Temperature

Optimum temperature of penshell juveniles was determined by measuring the scope for activity, and the rates of clearance, ingestion, and growth. Groups of five bivalves in triplicate were transferred to 19-L cylindrical tanks containing sea water at 38‰ and ambient temperature (22°C). The water of these tanks was gradually adjusted to the experimental temperatures (16, 19, 25, and 29°C) changing the temperature 1°C/day. Temperatures of 16 and 19°C were produced by dipping the tanks into a 1,100-L fiberglass tank, connected to a 1-hp titanium water chiller recirculating the water. In those treatments warmer than 22°C (25 and 29°C), a 200-Watt thermostatically controlled heater was introduced into each tank. Once all treatments were at the correct temperatures, a 10-day acclimation period was given, feeding the penshells a mixture of 3.6×10^7 cells/individual/day of *I. galbana* and *C. gracilis* in a 1:1 proportion. Half of the water volume was changed every day.

Scope for Activity

Scope for activity is the arithmetical difference between active and standard O_2 uptake rates. In this work, active respiration rates were measured by incubating, for 30 min, groups of five acclimated animals in 300-mL BOD bottles filled with O_2-saturated seawater at the experimental temperatures and 38‰ salinity, in triplicate. The volume of the bottles was determined at each experimental temperature by calculating the difference between the weight of the bottle filled with distilled water, minus the weight of the empty bottle. The seawater was previously filtered through a 0.75-μm GF/F membrane to reduce respiration from other organisms. Three bottles treated in the same manner without animals served as controls. After incubation, the oxygen content (PO_2) of the bottles was accurately estimated by determining the PO_2 of four subsamples of water siphoned into ground-neck borosilicate glass tubes of approximately 7 mL. Subsamples were fixed for further O_2 determinations following the method of Maeda-Martínez (1985). This method uses a miniaturized version of a micro-Winkler titrator developed by Bryan et al. (1976). This titrator has been used by Sicard et al. (1999) with good results.

Active O_2 uptake rate (AVO_2) was calculated with the equation

$$AVO_2 = \frac{PO_{2i} - PO_{2f}}{\Sigma DTW \times 0.5\ h}$$

where PO_{2i} is the oxygen concentration in the controls, PO_{2f} is the oxygen concentration in the incubated bottles, Σ DTW is the dry tissue weight of juveniles incubated, and h is the incubation time in hours. Once the active rates were determined, the animals were transferred to their original acclimation tanks, and were kept at the same conditions as before, but without the addition of food. The VO_2 of the organisms from all treatments was frequently measured, observing a gradual decline in VO_2, until a minimum stable level was reached. This level was equal to the standard respiration rate (SVO_2). The scope for activity of the penshell juveniles was calculated by $AVO_2 - SVO_2$ at the different temperatures tested. A one-way analysis of variance (ANOVA) was done to find significant differences of AVO_2 and SVO_2 at different temperatures. A Tukey's test was applied to find similarities among temperature treatments. Optimum temperature was where the difference was a maximum.

Ingestion and Clearance Rates

Ingestion rates (IR) and clearance rates (CR) were measured following the principle of the method of Winter (1973). This estimates IR and CR from the amount of algae removed by the animals incubated at a constant algal concentration. In this way variations on feeding rates because of a decrease in food concentration are eliminated. The amount of algae cleared by the animals is calculated from the algae replenished into the incubating chamber as removal occurs. Replenished algae will be equivalent to the algae consumed. In the present work we used a system that operates under this principle (Sicard et al. 1999) (Fig. 1), based on the design of Gallager and Mann (1980). Five juveniles of *A. maura* were incubated in a 1-L cylindrical chamber containing seawater filtered at 7 μm at the same experimental temperatures of the scope for activity experiments, 38‰ salinity, and 8×10^4 cells/mL of *I. galbana*. The water was pumped to a flow-through cell of a Turner 112 fluorometer with a peristaltic pump at 87 mL/h. The fluorometer was optimized for chlorophyl *a* readings by using a 47B filter and 420–500 nm wavelength. The relationship between microalgae concentration (MC) (cells/μL) and the fluorometer output voltage (Volts) was described by the linear regression equation ($R^2 = 0.98$; $n = 10$)

$$MC = 226 \times Volts - 22.3$$

The water from the cell was returned to the incubation chamber. When the algal concentration dropped, the system operated a second peristaltic pump which transferred concentrated microalgae to the chamber at 1.0 mL/min until the concentration was reestablished. The algae in the chamber were kept homogeneous in suspension with an air bleed. The system recorded the volume of algae pumped (V) during a 2-h incubation time (t). IR (cells ingested/g DTW/h) was then calculated with the equation

$$IR = \frac{V \times S}{\Sigma DTW \times t}$$

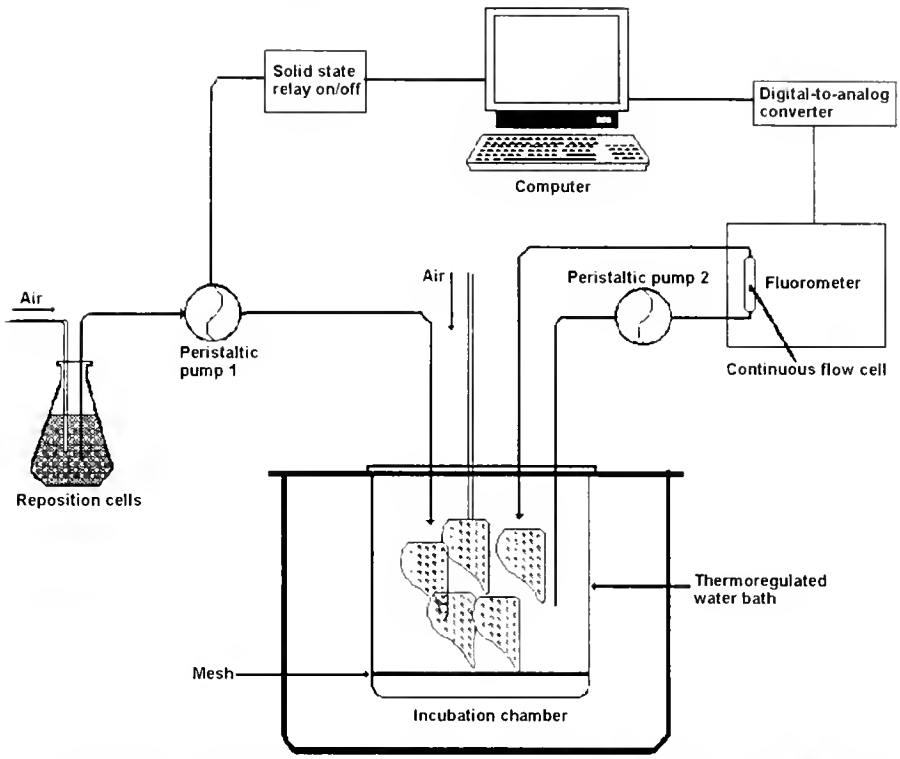

Figure 1. Apparatus employed for measuring clearance and ingestion rates in juvenile penshells (*Atrina maura*). Modified from Sicard et al. (1999).

where S was the concentration of microalgae in the replenishing stock.

From this, CR (Liters of water/g DTW/h) was calculated with the equation

$$CR = \frac{IR \times \text{Incubation chamber volume (L)}}{\text{Algal concentration in incubation chamber}}$$

The experiments were run in triplicate at each experimental temperature. An ANOVA was done to find significant differences of IR and CR at different temperatures. A Tukey's test was applied to find similarities among temperature treatments.

Growth and Survival

Growth and survival of penshell juveniles were recorded for 42 days. Groups of eight animals (18.7–21.0 mm shell height) were placed in tanks containing 19-L aerated seawater, 38‰ salinity and at the same experimental temperatures as in the previous experiments. The animals were fed $1.8 \pm 0.6 \times 10^8$ cells/individual/day of a mixture of *I. galbana* and *C. gracilis* at equal proportions. Half of the water was changed every day. Shell height was measured with plastic calipers every seven days over 42 days. Survival was measured daily, removing the dead animals from the tanks to avoid contamination of the water.

RESULTS

Dry Tissue Weight

The relation between dry tissue weight (DTW) and shell height (SH) of penshell juveniles is shown in Figure 2 and is described by the equation ($r = 0.95$; $n = 24$)

$$DTW = 2.3 \times 10^{-6} \times SH^{3.5}$$

Halotolerance

LD_{50}-96 of penshell juveniles to salinity is shown in Figure 3. Results show that the penshell withstands a range of salinity from 16.5‰ to more than the highest salinity tested (50‰) in the present study. As 85% mortality was only obtained at 50‰ salinity at 96 h, the median lethal time was calculated instead, extending the experiment until half of the population died at this salinity. Results indicate that the median lethal time for penshell juveniles was at 168 h (7 days) at this salinity (Fig. 3).

Thermotolerance

The upper temperature LD_{50}-96 of penshell juveniles is shown in Figure 4. Results indicate that *A. maura* tolerates high tempera-

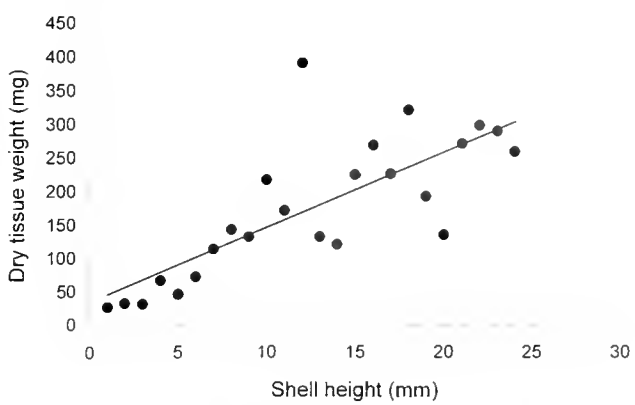

Figure 2. Relation between shell height and dry tissue weight in juvenile penshells (*Atrina maura*).

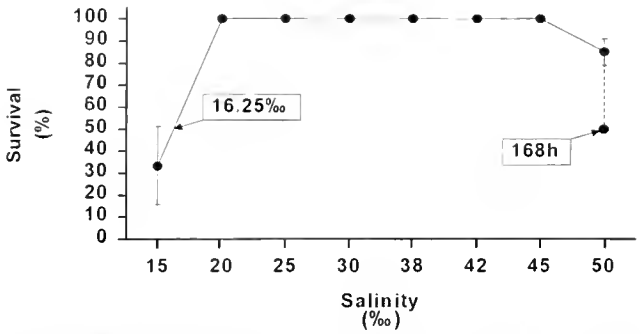

Figure 3. Median lethal dose at 96 h (LD_{50}-96) of salinity, on penshell (*Atrina maura*) juveniles. Because LD_{50} was not obtained at 96 h and at the highest salinity tested (50‰), the median lethal time was determined (broken line). Bars are the standard deviation of the means. $n = 3$.

tures, with the LD_{50}-96 of 33.2°C. Survival was not affected between 19 and 30°C, and at 32°C only 10% mortality was recorded.

Scope for Activity

Active (AR) and standard (SR) respiration rates at 16, 19, 25, and 29°C are shown in Figure 5. Results indicate that AR rates varied directly with temperature from 0.65 $mLO_2/g/h$ at 16°C, to 1.15 $mLO_2/g/h$ at 29°C. However SR rates remained stable between 0.2 and 0.4 $mLO_2/g/h$ and independent of temperature. SR was reached within 15 and 20 days from starvation in all treatments. With these results, the scope for activity (AR to SR) (Fig. 6) increased with temperature from 0.4 $mLO_2/g/h$ at 16°C to 0.9 $mLO_2/g/h$ at 29°C. The ANOVA indicated significant differences between temperature treatments at $P > 0.05$. However the Tukey's test revealed significant differences between all treatments but not between 16 and 19°C. No inflection of the curve was observed even at higher temperatures tested, which did not allow the determination of the temperature optimum in this species.

Ingestion and Clearance Rates

IR and CR of penshell juveniles are shown in Figures 7a and 7b. Results indicate that both rates increased with temperature, in the same manner as the scope for activity. Mean IR increased from 2×10^7 cells/g/h at 16°C, to 6.5×10^7 cells/g/h at 29°C. CR varied from a mean of 0.6 L/g/h at 16°C, to 2.25 L/g/h at 29°C. Large variations of IR and CR were observed between replicates of the all treatments, as indicated by the large standard deviation bars in

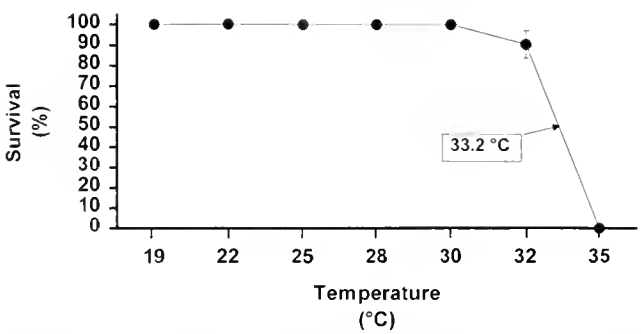

Figure 4. Upper median lethal dose at 96 h of temperature on penshell (*Atrina maura*) juveniles. Bars are the standard deviation of the means. $n = 3$.

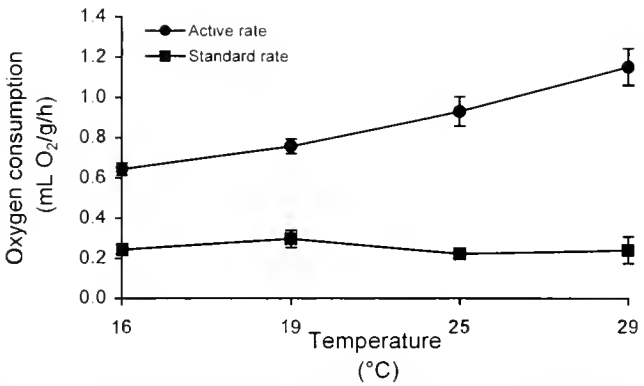

Figure 5. Active and standard respiration rates of penshell (*Atrina maura*) at different temperatures. Bars are the standard deviation of the means. $n = 3$.

Figures 7a and 7b. ANOVA indicated no significant differences of IR and CR at different temperature treatments ($P > 0.05$).

Growth and Survival

The results of growth experiments at 16, 19, 25, and 29°C are shown in Figure 8. Results indicate that growth in penshell juveniles correlated with temperature. Linear shell height increments of 0.021, 0.029 and 0.058 mm/day were obtained at 16, 19, and 25°C. However at 29°C, the height increment was low (0.5 mm) during the first 14 days, and then increased rapidly to 2.9 mm in 42 days. No explanation for this finding was found. Mortality was not recorded during the 42 days of experimentation in all treatments, with the exception of a slight decrease in survival (15%) on day 21 at 29°C.

DISCUSSION

The results indicate that *A. maura* is a tropical species that withstands a large range of salinity and high upper temperatures. Because of this, *A. maura* appears to be suitable for aquaculture in a wide variety of environments from hypersaline lagoons like those in Baja California, estuaries located along the Pacific coast of Mexico, Central, and South America, and probably in earthen ponds similar to those used in shrimp aquaculture. With our findings, temperature does not seem to impose a limit in the selection of sites, because the upper thermotolerance of penshells is greater than the maximum temperatures recorded in most bays of the Pacific coast of Mexico and of the Gulf of California (Lluch-Cota et al. 2000). In the present work, optimum temperature for growth

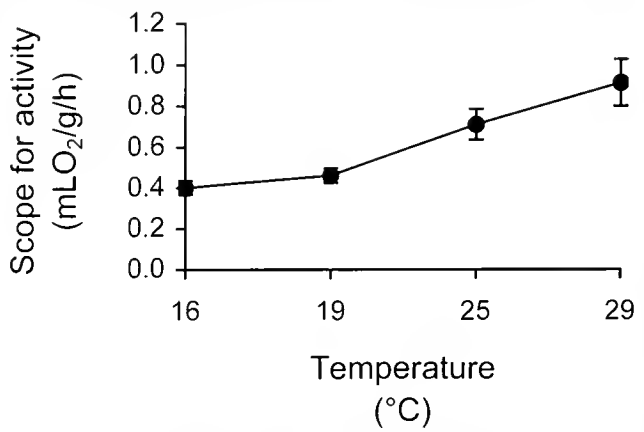

Figure 6. Scope for activity of juvenile penshells (*Atrina maura*) at different temperatures. Bars are the standard deviation of the means. $n = 3$.

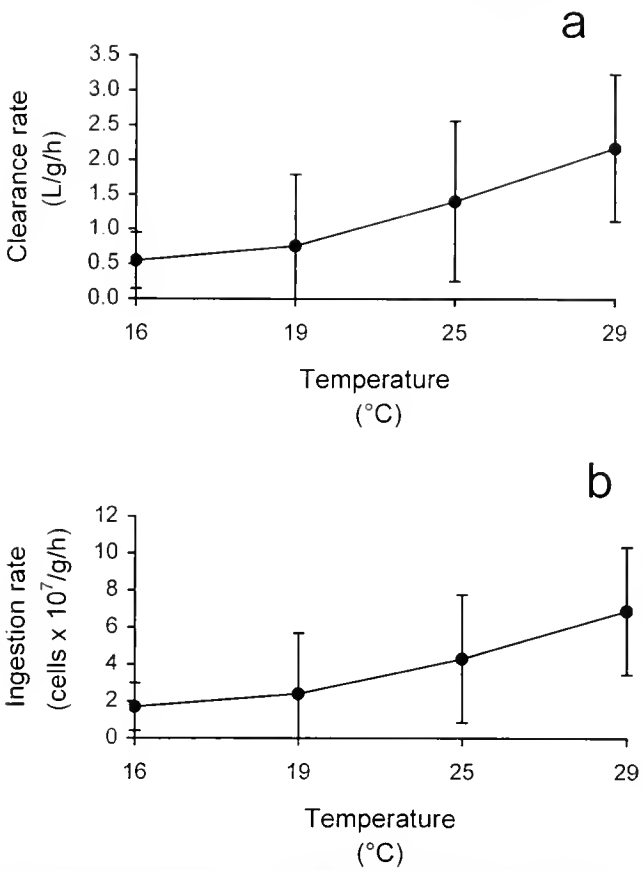

Figure 7. Clearance (a) and ingestion (b) rates of penshell (*Atrina maura*) juveniles at different temperatures. Bars are the standard deviation of the means. $n = 3$.

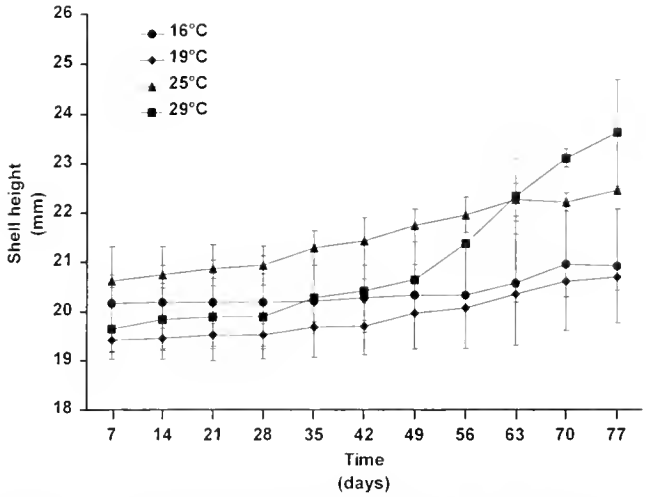

Figure 8. Growth of penshell (*Atrina maura*) juveniles, cultured at different temperatures, 38‰, and fed $1.8 \pm 0.6 \times 10^8$ cells/individual/day of a mixture of *Isochrysis galbana* and *Chaetoceros gracilis* at equal proportions. Values are the mean of 8 individuals ± standard deviation.

in penshell juveniles was not found within the range of temperatures studied. This could probably have been found if a higher temperature was tested. However, optimum temperature in *A. maura* is close to 29°C, because apart from the higher growth rate measured, reproductive maturation in the laboratory is faster at 25 and 30°C than at lower temperatures (Rodriguez-Jaramillo et al. 2001). Scope for activity and feeding rates gave identical results and coincided with growth experiments. This confirms the findings of previous work in catarina scallop (*Argopecten ventricosus*) (Sicard et al. 1999) where scope for activity, IR, CR, and growth gave similar results indicating an optimum temperature for growth of 19–22°C, in the range of temperatures tested (12–28°C). Therefore, any of the methods studied here appears to be adequate for the determination of optimum temperatures in molluskan species. However precision of feeding rates is lower than scope for activity and growth rates. Our ingestion and clearance rates experiments showed large variations between replicates of the same treatment. Variations do not seem to result from technical problems, but from a discontinuous feeding behavior of the animals. Large dispersion of IR and CR data have also been reported in *Argopecten irradians* (Kirby-Smith 1970), *Mytilus edulis* (Widdows and Bayne 1971) and in *A. ventricosus* (Sicard et al. 1999).

In contrast, Thompson and Bayne (1972), Bayne (1973), Bayne et al. (1973), Widdows (1973), and Sicard et al. (1999) have considered the scope for activity as a practical way to find the environmental conditions at which the energy available to the organism for growth and for other vital functions is a maximum. Both active and standard respiration rates are normally dependent on temperature as seen in *M. edulis* (Bayne 1976) and *A. ventricosus* (Sicard et al. 1999). However in our work, only the active rate varied with temperature. Standard respiration rate remained fully independent of temperature, which is not common. This is only found when insensitivity to temperature change may help to conserve metabolic energy reserves (Bayne 1976), as in situations of environmental stress (extremes of temperature, during starvation, or when exposed to air at low tide). Further work is needed to clarify these findings.

In the present work, salinity and upper temperature tolerance limits of penshell juveniles have been determined. We have demonstrated that the optimum temperature for growth in this species is 29°C or higher, making *A. maura* a good candidate for aquaculture in subtropical and tropical waters.

ACKNOWLEDGMENTS

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AN *IN VITRO* ASSAY TO DETECT PARALYTIC SHELLFISH POISON

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ABSTRACT We have developed an *in vitro* assay named Paralytic Shellfish Poison-test (PSP-test) that is capable of detecting low quantities of saxitoxin (STX), the first toxin studied from PSP. The PSP-test is an immunoassay based on the development of specific monoclonal antibodies (MAbs) capable of recognizing soluble saxitoxin (STX) and able to agglutinate in minutes, a latex containing STX adsorbed to its surface. The PSP-test is a two-step indirect immunoassay. During the first step, the MAbs are incubated with an extract suspected of containing STX. If the solution contains STX, this molecule will occupy the MAbs binding sites and block the latex agglutination. In the second step, MAbs-STX are incubated with an STX-latex. An inhibition indicates the presence of STX (PSP) in the sample. The PSP-test can detect within the range of 1.25–2.5 μg/ml of STX standard. It is also capable of recognizing STX in whole shellfish extract, acidic shellfish extract, and supernatants from aged toxic cultures of bacteria or dinoflagellates. The assay is simple, economic, and quick. Training of in its use is fast and harmless to the user. It can be performed in the field as a first screening procedure because an instrument to interpret the results is not required. However, the PSP-test was not developed to replace the mouse bioassay but rather to be complementary, and the strategy used for its development can be easily adapted to other toxins.

KEY WORDS: Paralytic shellfish poison, saxitoxin, monoclonal antibodies, dinoflagellates, assay

INTRODUCTION

The world oceans are affected by temporary massive proliferation of microalgae called Harmful Algal Blooms (HAB), commonly known as "Red Tides." Among the toxins produced by these HABs, the most feared is the Paralytic Shellfish Poison (PSP), a neurotoxic toxin produced by dinoflagellates like *Alexandrium, Gymnodinium* and *Pyrodinium*. There have been reports that some bacteria like *Anabaena, Aphanizomenos, Moraxella* and *Pseudomonas* are capable of producing PSP (Rausch and Lassu 1991).

Monitoring PSP toxicity potential in shellfish is carried out using the mouse bioassay, the only international assay accepted for certification of shellfish safety (AOAC 1990). The mouse bioassay has a detection limit of about 40 μg of STX for 100 g of total shellfish tissue. However, reports indicate that this assay has high variability (Park et al. 1986) and the maximum concentration of STX accepted for shellfish commercialization (80 μg/100 g) is too close to the limit of the mouse detection assay. This situation has stimulated the development of new methods for detection of toxin concentrations lower than those detected by the mouse bioassay. Among the new assays is high pressure liquid chromatography (HPLC) which is one of the most versatile and sensitive (Sullivan et al. 1983., Oshima et al. 1984). The fact that STX is able to block the activity of the voltage-dependent sodium channel and displace radioactive STX bound to brain membranes by cold STX present in the shellfish extract, allows quantification of the STX (Davio and Fontelo 1984). Other methods also include the cytopathic effect of STX on neuroblastoma cells cultures (Kogure et al. 1988) and the use of monoclonal and polyclonal antibodies in several immunoassays formats (Chu and Fan 1985; Carlson et al. 1984; Davio, et al. 1985 and Usleber et al. 1991). The most accurate and reliable assay to detect PSP is liquid chromatography followed by mass spectrometric analysis, LC-MS (Quilliam et al. 1993). The main advantage of the assays listed above is, that they allow the evaluation of several shellfish samples simultaneously within a relatively short period of time when compared to the mouse bioassay. However, these assays can be performed only under laboratory conditions requiring expensive infrastructure and most of them cannot be easily adapted to field conditions as required to perform daily monitoring. Furthermore, the mouse bioassay is a time consuming and an expensive procedure. The main reason to maintain the mouse bioassay is that it detects the whole shellfish toxicity regardless of which toxin or toxin-complexes are present in the shellfish sample. On the other hand, animal rights defendants groups are encouraging and pressing the scientific community to look for alternative assays that will allow the elimination of the mouse bioassay or at least reduce the number of animals used.

Here, we describe an indirect immunoassay that detects low quantities of STX from a single shellfish sample. PSP-test is a qualitative assay, economical, easily adaptable to other toxins or toxic profile, and does not require instruments to interpret the results. Thus, the PSP-test can be adapted to field conditions allowing to rapidly alert shellfish farmers of the presence of contaminated shellfish and can be used as primary screening of samples before analysis of mouse bioassay.

MATERIALS AND METHODS

Generation of Monoclonal Antibodies (MAb)

STX was purchased from Sigma Chemical Company (St, Louis, MO). It was bound to hemocyanin ("blue carrier" protein), Biosonda (Santiago, Chile) to be used as immunogen and to bovine serum albumin (BSA) to be used as antigen for screening as described elsewhere by Levine et al. (1988)). Briefly, STX's hydroxyl group was modified using CMA (carboxymethylamine). It was then, incubated with a water-soluble carbodiimide EDC, followed by incubation for 1 h at room temperature with blue carrier

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protein. The ration STX-Protein was 100:1. Free STX was separated from the STX-protein conjugate by centrifugation through a microconcentrator Centricon™ 30 tube (Amicon, Danvers, MA). Similarly, STX was adsorbed to 1 μm latex following manufactures indications, Bangs Labs. (Fishers, IN). To immunize the animals, 50 μg of STX-blue carrier conjugate was mixed with Complete Freund Adjuvant, Gibco (Grand Island, NY) just before immunization. Female 10-week-old BALB/c mice were immunized subcutaneously. After the first immunization, four additional boosts were done at 20-day intervals. Five days after the last boost, serum samples were drawn from each mouse and their titers were determined by ELISA using polystyrene plates DK 4000, Nunc (Roskilde, Denmark) coated with the STX-BSA as antigen as described elsewhere by Chu (1985). The mouse with the highest titer for STX-BSA was used to generate the monoclonal antibodies.

After immunization, spleen cells from the selected mouse were fused with 2.5×10^7 NSO/2 mouse myeloma cells using 50% polyethylene glycol 4000 Merck (Rahway, NJ) as described by Köhler and Milstein (1975). Then, the cells were suspended in Dulbecco supplemented with 1% nonessential amino acids, 10% fetal bovine serum (FBS), HyClone Labs. (Logan, UT), penicillin (100 IU/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, 10^{-2} M hypoxanthine, 4×10^{-5} M aminopterin, and 1.6 μ 10^{-3} M thymidine, HAT selecting medium, Sigma, (St. Louis, MO). Aliquots of 100 μl were plated on 96-well tissue culture plates. The plates were incubated at 37°C in a moisturized atmosphere with 10% CO_2 in the air. ELISA, as described previously, screened 10 days after fusion, supernatants from cultures with hybridoma growth for specific monoclonal antibody secretion. All positive cultures were expanded onto 24-well plates, and cell samples were frozen in liquid nitrogen in a medium containing 90% FBS and 10% dimethylsulfoxide. Positive clones were re-cloned by limited dilution. Three hybridomas (8C3/A7, 6A4/H7 and 3F7) were selected for the test.

Shellfish Sample Extracts

Two types of shellfish samples were utilized for evaluation using the PSP-test. One, freshly frozen untreated shellfish from Aysén, XI region of Chile (43°45′ to 48°45′, Fig. 2 and Table 2). These same shellfish samples were subjected to acid extraction in performing the mouse bioassay (AOAC 1990). The other type was acidic shellfish extracts previously used to perform the mouse bioassay, obtained from different Chilean geographic areas affected by PSP.

Dinoflagellate Cultures

The OF875-17 clone of *A. tamarense* from Ofunato, Japan and clone GTCA28 of *A. fundyense* from New Hampshire, USA were

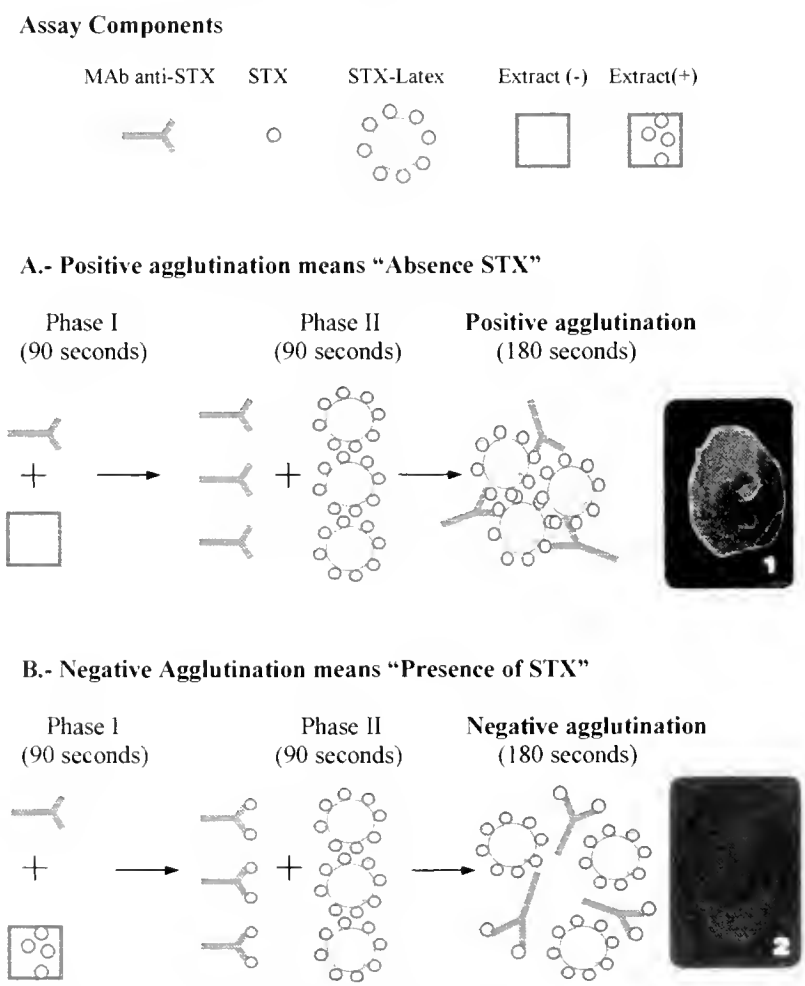

Figure 1. PSP-test assay detection principle. The latex agglutination indicates the absence of STX in the sample (A), while the inhibition indicates STX in the sample.

TABLE 1.

Assay detection limit and recognition of STX-isomers.

A Toxin	Concentrations				
Saxitoxin standard (μg/ml)	10	5	2.5	1.25	0.625
Agglutination	No	No	No	Yes	Yes

B Toxin	Concentration	Detection limit
Neosaxitoxin	28 μg/ampule	25 μg/1 mL
$GTX_{1/4}$ – 1	14.8/6.4 μg/ampule	39 μg/1 mL
$GTX_{2/3}$ – 1	24.8/5.8 μg/ampule	36 μg/1 mL
GTX_5 or B1*	~100 μg/ampule	200 μg/1 mL

Oligoclonal anti-STX antibodies cross-reactivity was assessed using commercially available STX and its isoforms. The lower STX concentration that induces inhibition of the agglutination was 1.7 μg/ml (A) and for the isoforms are as indicated (B).

* Indicates that this standard is not 100% pure.

kindly donated by Dr. Donald M. Anderson, Biology Dept., Woods Hole Oceanographic Institution, MA, USA. The ACC07 clone of *A. catenella* was isolated on April 1994 from Costa Channel, XI region, Chile (45°32′S, 73°34′W), and was kindly donated by MSc. Miriam Seguel, IFOP, Puerto Montt, Chile. Dinoflagellate cultures were maintained in glass tubes at 10°C with constant light until they reached stationary phase. Culture media consisted of sea water sterilized by passage through 0.2 μm filtration units Nalgene (Rochester, NY), supplemented with Guillard f/2 marine enrichment basal salts and vitamins (Sigma), as described by Guillard, (1975).

Bacterial Cultures

Pseudomonas diminuta was isolated from the ACC07 clone of *A. catenella*. The *Moraxella sp.* strain was isolated from *A. tamarense* and kindly provided by Dr. Masaki Kodama, School of Fisheries Sciences, Kitasato University Sanriku, Iwate, Japan. Free-living bacteria identified as *Pseudomonas versicularis* and *Proteus vulgaris* were isolated from the Magellan Strait, Chile. All bacterial strains were grown in Zobell marine media and kept at 10°C (Kodama et al. 1988).

PSP-Test Assay Procedures

Determination of the Assay Detection Limit

Ten microliters of a 1:30 dilution of the oligoclonal anti-STX (a mixture of three MAbs that recognize STX) was mixed with 10 μl of? a serial dilution of STX standard in distilled water (Sigma) and placed over a spot in the agglutination plate, mixing for 90 seconds at room temperature. Then, 10 μl of STX-latex was added and mixed by gentle swirling of the agglutination plate until agglutination was visible in the control (without free STX). Also, the anti-STX cross-reactivity of the oligoclonal antibody mixture to STX-isoforms, neosaxitoxina (Neo-1), gonyautoxins-1 (GTX1/4-1), gonyautoxins-2 (GTX 2/3-1), and gonyautoxin-5 acetate (GTX-5 or B1), all obtained from the Certified Reference Materials Program, Institute for Marine Biosciences, National Research Council of Canada, were evaluated.

Evaluation of the Presence of STX in Freshly Frozen Shellfish

Samples were allowed to thaw at room temperature. Approximately 0.1 g of digestive individual gland was mixed in an Eppendorf tube with 100 μl of distilled water and minced with a pestle until the suspension was homogeneous. The tubes were then centrifuged at 14,000 rpm for 3 min (Eppendorf, Nathalar, Germany) and 10 μl of the supernatant was placed on the agglutination slide and evaluated, as described previously. Agglutination occurs in the control spot about five minutes after the initiated reaction. The reaction for each sample was evaluated and scored as positive or negative for agglutination.

Evaluation of Whole Acidic Shellfish Extracts

Extracts from toxic and non toxic shellfish were obtained according to the AOAC procedure (AOAC 1990). Ten microliters of each sample was placed on the agglutination slide without neutralization and evaluated, as described previously.

Evaluation of Supernatants from Toxic Organisms

One milliliter of culture from stationary phase (10–12 days) either bacteria or dinoflagellate, was individually placed in an Eppendorf tube, after a quick spin to pellet the cells, 10 μl of supernatant was placed on the agglutination slide and evaluated, as described previously.

RESULTS

Assay Rationale

A mixture of MAbs (oligoclonal) generated against STX recognizes STX present in the latex surface (STX-latex) and agglu-

TABLE 2.

Evaluation of freshly frozen shellfish toxicity from Aysén, XI region of Chile by the PSP-test and the mouse bioassay.

Sample #	Shellfish		Mouse Bioassay		PSP-test
	Place	Species	Result	Death time or concentration	
1470	Melimoyu	chorito	N/D		+
1473	Melimoyu	almeja	N/D		+
1475	Seno Gala	chorito	+	1′:08″ 1′:26″ Sve.	+
1479	S. Gala	almeja	+	31 μg/100 g	+
1480	Seno Miller	culengue	+	0′:59″ 1′:07″ Sve.	+
1483	S. Miller	cholga	+	1′:04″ 1′:09″ 1′:08″	+
1484	S. Miller	chorito	+	1′:35″ 1′:51″ ′:02″	+
1485	S. Miller	almeja	+	42 μg/100 g	+
1486	Isla. Toto	chorito	+	2′:16″ 2′:00″ 1′:45″	–
1489	I. Toto	culengue	+	1′:25″ 1′:35″ 1′:25″	+
1490	I. Toto	almeja	+	55 μg/100 g	+
1491	Isla Manuel	chorito	+	2′:24″ 2′:07″ Sve.	+
1492	I. Manuel	cholga	+	1′:50″ 1′:54″ 2′:09″	–
1493	I. Manuel	almeja	+	59 μg/100 g	+
1496	Isla Gamal	almeja	N/D		+
1498	S. Magdalena	almeja	+	41 μg/100 g	+
1499	S. Magdalena	cholga	+	2′:07″ 2′:17″ 1′:55″	–
1502	Pta. Calqueman	culengue	+	1′:38″ 1′:37″ 1′:38″	–
1504	Pta. Calqueman	almeja	+	3′:11″ 3′:25″ 2′:50″	+
1505	Pta. Calqueman	chorito	+	1′:08″ 1′:15″ 1′:31″	–

If two out of three mice died, the sample was considered positive.

N/D: Non-detectable or negative using mouse bioassay.

Sve: Survival.

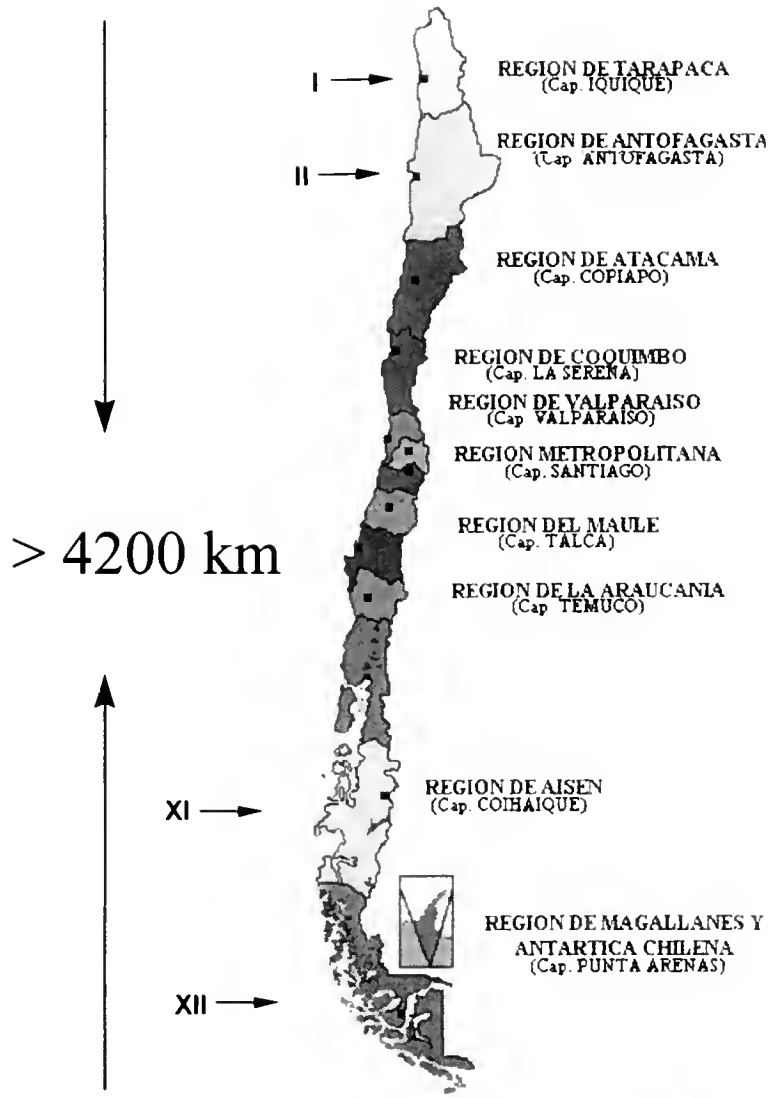

Figure 2. Geographical origin of Chilean shellfish samples used in this paper (Regions I, II, XI, and XII).

tinates it. The MAbs should not agglutinate the STX-latex if their binding sites are occupied by STX present in the sample (Fig. 1). Thus, if a sample, that does not contain STX, is mixed with the anti-STX MAbs, their binding sites will remain free and able to agglutinate the STX-latex. This positive agglutination indicates the absence of STX (PSP) in the sample. However, if the sample does contains STX, then the MAbs binding sites will be occupied and would not be able to agglutinate the STX-latex. Thus, the lack of agglutination indicates the presence of STX (PSP) in the sample.

Assay Detection Limit and Recognition of STX-Isomers

The detection limit was established as the minimal concentration of standard STX that inhibits agglutination after five minutes of reaction at room temperature. For STX this limit was in the range of 1.25–2.5 μg/ml (Table 1A). However, additional experiments with different STX sources indicate that this minimal inhibitory concentration may vary according to the sample condition, i.e. acid shellfish extract, culture, fresh, or canned (data not shown).

The PSP-test was evaluated to determine whether the MAbs recognize STX-isoforms. The MAbs anti-STX recognize different STX-isoforms evaluated as indicated in Table 1B. The STX-isoforms concentration that inhibits the agglutination is greater, as expected, compared to that of STX from monoclonal antibodies raised and screened for that purpose.

Analysis of Fresh Frozen Samples

Once the PSP-test assay conditions were established, we were interested in determining whether the assay can detect PSP in individual non-chemical extracts from freshly frozen samples. The results are presented in Table 2. Also, the same samples were used to detect PSP using the mouse bioassay with 3 mice of the F-1 strain. Death times and PSP concentration calculations are included in Table 2.

Table 2 shows the results of the analysis done on 20 samples of 4 different shellfish species obtained from eight different places of XI region (Fig. 2). These frozen samples were all from one individual organism and stored under non-standardized conditions to

reflect field conditions more closely. Results obtained with the mouse bioassay and with the PSP-test are compared in Table 2. The analysis shows that the mouse bioassay detected 17 (85%) positives out of 20 samples, and when the same shellfish samples were evaluated, the PSP-test detected 15 positives out of 20 (75%).

Analysis of Whole Acidic Shellfish Extracts

Whole acidic shellfish extracts from four different Chilean regions (Fig. 2) including eight different shellfish species from different years, were evaluated for PSP with the mouse bioassay, the data obtained are presented in Table 3. The same samples were evaluated with the PSP-test, giving a 100% of positive correlation with the mouse bioassay. Twenty-five samples of whole acidic shellfish extracts from non toxic shellfish (as determined by the mouse bioassay) gave negative results with the PSP-test assay (data not shown).

Analysis of Dinoflagellates and Bacterial Cultures

To determine whether the PSP-test assay could be used to detect toxic dinoflagellates reported as primary sources of PSP, we carried out the PSP-test in culture supernatants as well as in their supernatants obtained after cell homogenization. The results are shown in Table 4A. They indicate that the PSP-test detects PSP in both samples, regardless of which *Alexandrium* species we tested or where they came from.

In addition, the PSP test was shown as being capable of detecting toxins on culture supernatants from *P. diminuta*, a bacterium isolated from a Chilean strain of *A. catenella*, (Córdova et al., 2000). As positive control, we used a supernatant from cultures of *Moraxella sp.* a bacterium isolated from *A. tamarense* (Kodama et al. 1988). The confirmation that the bacteria isolated produced STX was done by HPLC (data not shown). Finally, several free-living marine bacteria isolated from XII region, Chile, were also evaluated for PSP production using the PSP-test. The results indicate that only two isolates, *P. versicularis* and *P. vulgaris*, were capable of producing PSP.

DISCUSSION

The mouse bioassay is used to monitor shellfish toxicity for legislative purposes (open or closing areas for shellfish harvesting), as well as to evaluate shellfish wholeness, fresh or canned, before commercialization and human consumption. Thus, evaluation of shellfish toxicity has been the best alternative to mitigate the negative impact of PSP poison in the community.

Despite the effectiveness over the years of the mouse bioassay in detecting shellfish toxicity, there are problems with accuracy

TABLE 3.

Toxicity detection in acid extracts of shellfish species harvested from different geographical areas. Samples evaluated by the mouse bioassay and the PSP-test as indicated under Materials and Methods.

Geographic location region	Shellfish			Mouse (µg/100 g)	PSP-test
	Sample #	Species	Place		
I and II					
03-12-1997	524	ostión	Caleta Las Verdes de Iquique	+65	+
05-02-1998	118	locos	Litoral Iquique	+37*	+
09-02-1998	123	locos	Punta Atala, Tocopilla	+49	+
15-05-1998	293	machas	Sector Playa Machas	+34*	+
	294	ostión	Sector Lagunilla	+35*	+
XI					
13-05-1998	1817	cholga	Seno Gala	+402	+
	1821	culengue	Seno Miller	+2875	+
	1831	culengue	Isla Toto	+3875	+
	1845	chorito	Caleta Puyuhuapi	+471	+
	1847	picoroco	Islas Orestes	+157	+
	1850	almeja	Islas Orestes	+316	+
	1867	picoroco	Islas Huichas	+108	+
	1870	cholga	Islas Huichas	+2297	+
	1879	cholga	Puerto Amparo	+651	+
	1881	chorito	Puerto Amparo	+445	+
XII					
02-03-1994	18	chorito	Bahía Porvenir, Tierra del Fuego	+1224	+
04-04-1994	601	cholga	Isla Isabel, Magallanes	+443	+
	655	chorito	Bahía Buena, Magallanes	+6516	+
	669	chorito	Cabo San Isidro, Magallanes	+3528	+
30-04-1994	878	cholga	Estero Nuñez, Magallanes	+601	+
07-07-1994	1304	cholga	Bahía Año Nuevo, U. Esperanza	+145	+
01-12-1995	996	almeja	Isla Magadalena, Magdalena	+137	+
19-04-1996	452	cholga	Bahía Stokes	+270	+
09-01-1997	17	chorito	Los Ñires, Magallanes	+1021	+
19-01-1998	115	chorito	Cabo San Isidro, Magallanes	+2011	+
	117	chorito	Cabo San Isidro, Magallanes	+2041	+

* Indicates that these values are slightly lower than the lower limit of the mouse bioassay test (Fernandez and Cembella 1995).

TABLE 4.

Evaluation of toxic cultures using the PSP-test.

A	Dinoflagellates species	PSP-Test Medium	PSP-Test Sup. Cell Homo.
	Alexandrium catenella clone ACC07, Chile	+	+
	Alexandrium fundyense clone GTCA28, USA	+	+
	Alexandrium tamarense clone F875-17, Japan	+	+

B	Bacteria Species		PSP-test Culture
1	*Pseudomonas diminuta*	Intracellular	+
2	*Moraxella* sp.-like	Intracellular	+
3	*Flavobacterium breve*	Intracellular	−
4	*Aeromona salmonicida*	Intracellular	−
5	*Pseudomonas versiculares*	Intracellular	−
6	*Pasteurella haemolyticus*	Intracellular	−
7	*Moraxella* sp.-like*	Intracellular	+
8	*Pseudomonas versicularis*	Free Living	+
9	*Proteus vulgaris*	Free Living	+
10	*Escherichia coli*	Free Living	−
11	*Pseudomonas mallei*	Free Living	−
12	*Vibrio parahaemolyticus*	Free Living	−

Bacterial and dinoflagellate cell cultures were evaluated detecting toxic dinoflagellate in their culture supernatant from stationary phase, as well as in their supernatants from cell homogenization (A).

Isolated bacteria from toxic dinoflagellate (1 and 2) as well as free living bacteria from the XII region of Chile (8 and 9) were detected positive for PSP production (B).

Other intracellular as well as extracellular bacteria tested negative for PSP production.

* PSP positive *Moraxella* sp. from Dr. Kodama.

and poor reproducibility when shellfish is contaminated with low concentrations of PSP. This indicates that there is a need for quick, sensitive, and economic assay, complementary to the mouse bioassay. The PSP-test could also be used for fast screening of toxic shellfish, allowing better management, hopefully, just before shellfish harvesting or to monitor toxic shellfish that undergo detoxification procedures, or shellfish acidic extracts before mouse bioassay, thereby reducing the number of mice used.

The PSP-test assay reported here has proven to be effective in detecting PSP in freshly frozen shellfish species, 15/20 (75%) when compared with positive samples in the mouse bioassay 17/20, (85%). The negative PSP-test results could indicate the presence of new STX isomers in the sample, different from those evaluated in Table 1B, which are not recognized by the MAbs anti-STX. However, this hypothesis, although possible, is unlikely because the death-times of the mice suggest high toxin concentration. Another explanation for this result could be that the shellfish among those pooled were diverse and possibly, not all contaminated, thereby resulting in different amounts of toxins in an individual assay. The mouse bioassay uses extracts from 100 g of shellfish; approximately 12 to 15 types of shellfish, increasing the chances of detecting toxicity. However, PSP-test uses an individual sample and a low amount of tissue. Finally, the possibility exists, although minimal, that some deaths might be due to a toxin that resembles PSP and consequently there would be no detection with PSP-tests. Unfortunately, samples to perform such, as HPLC or liquid chromatography-mass spectrometry, were not considered because we have no access to these technologies.

When the PSP-test was used in evaluating positive acidic shellfish extracts that were positive to the mouse bioassay, the results indicated 100% positive correlation. Therefore, based on this result, the PSP-test detects PSP under acidic conditions, suggesting that there is a potential for it to be used as a primary screening assay for these types of samples. Perhaps the number of mice used to evaluate PSP in shellfish acid extracts could be reduced. We believe that 100% correlation was obtained because toxins are concentrated from 100 g of shellfish. Regardless of what type of STX isoforms were present in the samples, under this acidic condition, they tend to transform to STX, isoform that is detected by PSP-test. The versatility of the PSP-test was also demonstrated when toxic cultures of dinoflagellates and bacteria were detected. However, a significantly higher number of samples will be necessary to assess the full potential of the PSP-test to detect PSP from different sources.

It is important to note that the PSP-test assay was not developed with the intent to replace the mouse bioassay, because it is incapable of recognizing "whole shellfish toxicity" as detected by the mouse (Table 2), which can be due to the highest standardization and reproducibility of sample preparation. However, the PSP-test has proven useful for detection of lower concentrations of PSP in fresh frozen shellfish, which indicates its potential use in testing field samples and for different shellfish species. The most important Chilean shellfish for local human consumption and for export were evaluated using the PSP-test. The following: "almeja del norte", *Prothaca thaca*; "almeja del sur", *Venus antiqua*; "chorito", *Mytilus chilensis*; "cholga", *Aulacomya ater*; "culengue", *Gari solida*; "loco", *Concholepas concholepas*; "macha", *Mesodesma donasium*; "ostión del norte", *Chlamys (Argopecten) purpuratus*; "picoroco", *Megabalanus psittacus*; indicates 9 out of these 19 species (47%) used. Moreover, the PSP-test can be improved by developing new MAbs characteristics to specific STX isomers present in the PSP, adapting it to different conditions and toxin profiles. Finally, the PSP-test uses a new technology that can be easily adapted for other shellfish toxins, and because it is economical and quick has the potential for uncomplicated shellfish monitoring.

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SPECIFIC INHIBITION OF ENDOGENOUS SHELLFISH PROTEIN PHOSPHATASE THAT COULD BE USED AS A DIRECT REPORTER OF DIARRHETIC SHELLFISH POISON

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ABSTRACT Diarrhetic Shellfish Poisoning (DSP) is a human gastrointestinal disorder associated with the consumption of contaminated shellfish. Okadaic acid (OA) and dinophysistoxin-1 (DTX-1) are diarrogenic DSP toxins, which also have tumor promotion and protein phosphatase inhibition activities. It is also known that OA and DTX-1 toxins localize mainly in the shellfish digestive gland; although, it remains to be explained how the toxins are retained in this tissue. Total protein of shellfish digestive gland from different shellfish species was fractionated using gel electrophoresis under nondenaturing conditions followed by specific phosphatase colored reaction. This procedure allows the identification of endogenous shellfish protein phosphatases that are shellfish species specific. Moreover, when shellfish digestive glands naturally contaminated by DSP were evaluated using the same procedure, one or both of previously identified endogenous protein phosphatase activities showed reduction or inhibition as compared to uncontaminated controls. To demonstrate experimentally that the protein phosphatase inhibiton observed were attributable to DSP toxins, shellfish of *Venus antiqua* were toxified using *Prorocentrum lima* cells under laboratory conditions, and their digestive glands were analyzed as described above. The same protein phosphatase inhibition was detected in shellfish naturally contaminated with DSP. These observations suggest that endogenous shellfish protein phosphatases could act as acceptors for DSP toxins, and their analysis can be used as a simple and sensitive assay to detect DSP toxins in shellfish, avoiding DSP purification steps, and reducing the time for DSP detection and animal use.

KEY WORDS: diarrhetic shellfish poison (DSP) detection, shellfish acceptors for DSP toxins, endogenous shellfish protein phosphatase detection, DSP monitoring

INTRODUCTION

Dinophysis sp. and *Prorocentrum* sp. are marine dinoflagellates that produce toxins that cause diarrhetic shellfish poisoning (DSP) in humans after consumption of contaminated shellfish. Yasumoto et al. (1979) was the first to describe this syndrome in which *Dinophysis fortii* was identified as the biological source for DSP toxins found in contaminated shellfish in Japan (Yasumoto et al. 1980). Since its discovery in Japan, DSP syndrome has been reported in other countries worldwide (Maestrini, 1998).

Among the DSP toxins, okadiac acid (OA) and dinophysistoxin-1 (DTX-1) can cause diarrhea, and both also are tumor promoting agents and inhibitors of protein phosphatases (Yasumoto et al. 1985, Takai et al. 1987, Fujiki et al. 1988). Although most countries accept up to 200 ng of OA per g of tissue, there is a general consensus that only shellfish free of OA and DTX-1 for human consumption and international commercial trade should be acceptable.

Shellfish probably become contaminated by concentrating DSP toxins while feeding on marine phytoplankton (Murakami et al. 1982, Bauder et al. 1996, Quilliam et al. 1996). Moreover, several assays have been developed to detect smaller quantities of OA and DTX-1 in shellfish samples, especially because of their tumor-promoting activity. The assays to detect DSP toxins can be grouped into two sets. The first set includes assays that can detect such DSP biological activity as the mouse bioassay (Japanese Ministry of Health and Welfare 1981), effects on rat appetite (Terao et al. 1993), dehydration effects on the newborn mouse intestine (Hamano et al. 1986), and cytophatic effects on cultured cells (Amzil et al. 1992). In addition, a colorimetric assay was developed based on the ability of OA to inhibit phosphatases PP-1, PP-2A, and PP-2B (Shikari et al. 1985). The second set of assays detects DSP active molecules and their isoforms or intermediary molecules. These are enzyme-linked immunosorbent assay (ELISA) (Uda et al. 1989); radioimmunoassay (RIA) (Levine et al. 1988), high-power liquid chromatography (HPLC) (Lee et al. 1987), and the most sophisticated assay, liquid chromatography followed by a mass spectrometry (LC-MS/MS) (Pleasance et al. 1990). However, all of these assays require several steps of purification before they are performed. Clearly, a simpler and economical assay is needed to monitor DSP toxins in shellfish before harvesting and to reduce the number of mice used in the bioassay, because of its poor reproducibility, high number of false-positives, and the excessive time required to determine toxicity (Tagaki el al. 1984, Kogawa et al. 1988).

Here, we report that endogenous shellfish protein phosphatase activity localized in digestive glands can be easily identified using gel electrophoresis (SDS-PAGE) under nondenaturing condition followed by specific colored reaction. Using this procedure, it was possible to demonstrated that protein phosphatase activity from three different species of shellfish naturally contaminated with DSP are specifically disminished or inhibited, as compared to their uncontaminated control samples. Moreover, DTX-1 was detected by LC-MS when the gel band containing the inhibited protein phosphatase was analyzed. Furthermore, protein phosphatase inhibition similar to DSP natural contaminated shellfish, was observed when *Venus antiqua* was toxified with *Protocentrum lima* cells under laboratory conditions. Finally, protein phosphatase activity or its inhibition can be detected in as little as 100 μg of total protein from aqueous extract of digestive tissue gland without purification steps. These observations suggest that endogenous shellfish protein phosphatases localized in the digestive gland act as DSP aceptors. Furthermore, analysis of the endogenous shellfish protein phosphatase condition (active or inhibited), may be used as a simple and sensitive assay to detect DSP toxins in shellfish, avoiding DSP purification steps, reducing the time for DSP detection and animal use.

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MATERIAL AND METHODS

Microalgae Cultures

The Canadian strain of toxic *Prorocentrum lima* was kindly provided by Dr. Allan Cembella (Institute for Marine Biosciences, National Research Council, Nova Scotia, Canada). Microalgae were cultured at 15°C in f/2 medium (Guillard 1975) and were exposed to continuos illumination provided by day-like type fluorescence light. Cultures of the nontoxic microalgae used to feed shellfish were: *Isochrysis galbana*, *Isochrysis suecica*, *Nannochloris* sp., and *Pavlova* sp. were kindly provided by Dr. Gloria Coyantes (Montemar, Valparaíso University, Chile) and maintained with the same conditions as for the *P. lima* culture. Three-week-old cultures of *P. lima* were used to toxify shellfish.

Evaluation of Endogenous Shellfish Protein Phosphatase Activity

Positive DSP frozen digestive glands of different shellfish species *Aulacomya atter*, *Mytilus chilensis*, and *Venus antiqua* were from the Chilean Monitoring Program and kindly donated by Ms. Georgina Lembeye. These samples were evaluated using the mouse bioassay (Yasumoto 1984, Japanese Ministry of Health and Welfare 1981), although exact concentration of DSP was not determined. Uncontaminated shellfish species of *A. atter*, *M. chilensis*, and *V. antiqua* for control were bought from a local seafood market. Frozen tissues were thawed on ice. Then, 0.1 g of each tissue was individually mixed with 200 μL of sterile distilled water, minced, and centrifuged at 14,000 rpm for 5 min using a benchtop centrifuge. Protein concentration was determined using the BioRad DC kit (Richmond, CA), and adjusted to 2 mg ml^{-1}. Fifty μL of the aqueous extract was mixed with 50 μL of SDS-PAGE sample buffer (0.0625 M Tris-HCl, pH 6.8; 10% glycerol, 2.3% sodium dodecyl sulfate (SDS), and 0.00125% of bromophenol blue). The sample was fractionated without heat denaturation using 8% sodium dodecyl sulfate-polyacrylamide gel (7.5 × 10 cm) electrophoresis (SDS-PAGE) according to Laemmli (1970) in a Mini Protean II Electrophoresis Cell (BioRad). The prestained molecular weight marker proteins were from New England BioLabs (Beverly, MA). The gel run at 60 V for 2 h using 0.025 M Tris-HCl pH 8.8; 0.192 M glycine, and 0.0035 M SDS as running buffer. The gel was then washed three times with distilled water for 5 min each at room temperature. Next, the gel was incubated with alkaline phosphatase buffer (100 mM Tris-HCl pH 9.5; 5 mM MgCl2; 100 mM NaCl) for 10 min at room temperature. The buffer was then replaced and supplemented with 4.3×10^{-4} M of 5-bromo-4-chloro-3-indolyl phosphate and 3.9×10^{-4} M of nitro blue tetrazolium (BCIP-NBT, Sigma Chemical, St. Louis, MO) dissolved in 100 and 70%, respectively of N,N-dimetylformamide (Merck, Darmstadt, Germany), and incubated at room temperature until precipitated bands were visualized. After the bands were clearly identified, the gel was washed twice with distilled water and scanned using the AGFA studioscan II Si connected to an Apple Macintosh Performa 5300 computer.

Detection of Protein Phosphatase Activity in Shellfish Contaminated Under Laboratory Conditions

Four live *V. antiqua* shellfish from a local seafood market were washed with fresh seawater and placed onto a 1-L beaker containing 600 mL of fresh seawater. The beaker was maintained at 10°C, and also air was bubbled constantly into it using a fish tank pump. Shellfish viability was confirmed when the shells opened, and water circulation was observed through the siphons. Shellfish were fed daily for seven days as follows: air bubbling was stopped; 2 mL containing of 10^5 cells ml^{-1} of a three-week-old *P. lima* culture were added to the beaker. Ten minutes later, 5 mL containing a mixture of 10^6 cells ml^{-1} of viable nontoxic microalgae *I. galbana*, *I. suecica*, *Nannochloris* sp., and *Pavlova* sp was added to each beaker. After 30 min, air bubbling was reinitiated. After the seventh day, digestive glands were individually removed from each group and individually analyzed by SDS-PAGE, as described above. The seawater was changed once during the seven-day experiment. These experiments were carried out with aged *P. lima* cultures, because it has been shown that older cultures are more toxic (Quilliam et al. 1996).

Analysis of the SDS-PAGE Band Containing the Inhibited Protein Phosphatase

Ten milligrams of protein aqueous extract from *V. antiqua* digestive gland DSP naturally contaminated were fractionated by SDS-PAGE (15 × 17 cm), as described above. The gel piece containing the inhibited protein phosphatase band (using as reference the prestained molecular weight markers) was cut off and subjected to three washes with distilled water. The gel piece was then minced and mixed with 5mL of 50% methanol, and vortexed for 10 min at room temperature. The gel fragments were then also subjected to constant sonication for 2 min using the lower power of a Sonifier 450 (Branson Ultrasonic, CT). The sample was centrifuged at 2,500 rpm for 10 min and the supernatant concentrated at 65°C until 1 mL of volume; it then was completely dried out using a speed-vac system. The sample was sent to Institute for Marine Biosciences, National Research Council of Canada, in Canada, for analysis by liquid chromatography-mass spectrometry (LC-MS) according to Pleasance et al. (1992) to determine which DSP toxin was present in the gel band.

RESULTS

Protein phosphatase activities from three different shellfish species were characterized by SDS-PAGE followed by phosphatase specific staining that produces a precipitated colored band (Fig. 1). Uncontaminated *A. atter* and *M. chilensis* had two bands at 85 and 150 kDa (Fig. 1, Lanes 1 and 3); *V. antiqua* has two bands at 70 and 140 kDa (Fig. 1, Lane 5). The two bands in *A. atter* and *M. chilensis* showed similar protein phosphatase activity as deduced from the intensity of their bands, which was optically assessed. In contrast, in *V. antiqua*, the higher band (140 kDa) had a greater protein phosphatase activity than the lower band (70 kDa).

On the other hand, shellfish naturally contaminated with DSP toxins had their protein phosphatase activities significantly inhibited (Fig. 1, Lanes 2, 4, and 6. Thus, for *A. atter* and *M. chilensis*, the inhibition was similar and inhibited preferentially the higher band in both species (Fig. 1, Lanes 2 and 4). In contrast, *V. antiqua* had a different inhibition pattern: the lower band was almost totally inhibited, while the higher band seemed to have the same or more activity with respect to control band (Fig. 1, Lane 6).

Furthermore, analysis of the methanolic extract corresponding to the 70 kDa protein band from DSP naturally contaminated shellfish using the LC-MS analysis demonstrated the presence of DTX-1 at approximately concentration of 2 ng ml^{-1} (data not shown).

To confirm that inhibition of the 70 kDA protein phosphatase

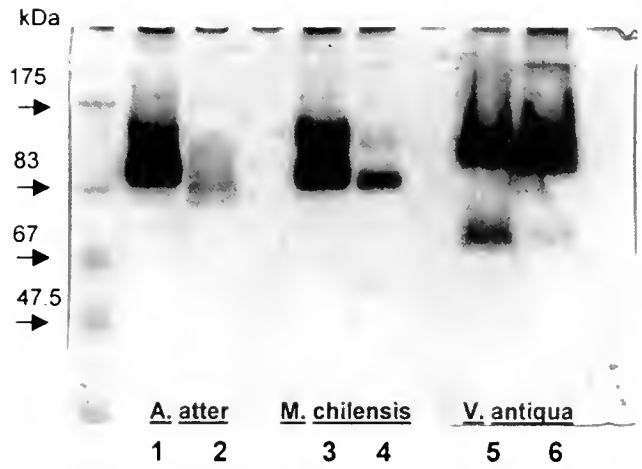

Figure 1. Shellfish phosphatase patterns. Phosphatase activities from three different shellfish species were determined by SDS-PAGE. Phosphatase activities from noncontaminated shellfish (Lanes 1, 3, and 5) are compared with shellfish naturally contaminated with DSP toxins (Lanes 2, 4, and 6). Molecular weight markers are as indicated.

band observed on naturally DSP-contaminated *V. antiqua* was attributable to DSP toxins, two groups of this shellfish were cultivated under laboratory conditions, only one group was fed with toxic *P. lima* cultures. The results are shown in Figure 2. Lanes 1 and 3 correspond to protein phosphatase activity of shellfish control that was fed only with nontoxic microalgae cultures. However, in this case, three protein phosphatase activity bands were visualized at 50, 70, and 140 kDa (Fig. 2, Lanes 1 and 3). Although in contaminated samples (Fig. 2, Lanes 2 and 4), the 140 kDa showed an increased protein phosphatase activity it when was compared to the same band in the noncontaminated shellfish. The 50 and 70 kDa bands were inhibited.

DISCUSSION

This work reports that endogenous shellfish protein phosphatases localized in digestive glands undergoes specific inhibition as result of contamination with DSP toxins. Protein phosphatase activity can be visualized after separating them from the shellfish digestive gland using SDS-PAGE nondenature condition and exposure to phosphatase-specific substrates that precipitated upon

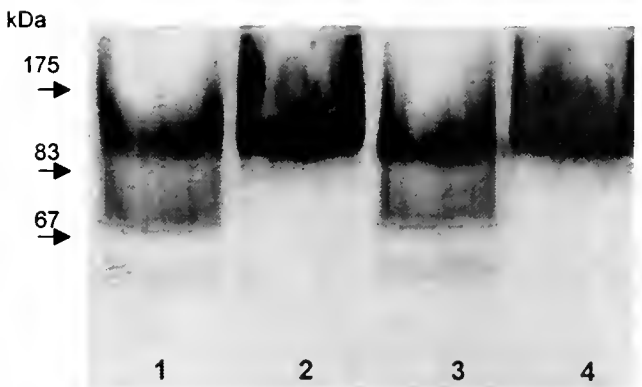

Figure 2. Inhibition of *V. antiqua* phosphatases. Phosphatase activity is inhibited when shellfish were fed with toxic *P. lima* (Lanes 2 and 4) as compared with control shellfish (Lanes 1 and 3). Molecular weight markers are as indicated.

phosphatase activity. The intensity of the stained band is proportional to the number of phosphatase molecules present in the gel band. Thus, Figure 1, Lanes 1, 3, and 5 show the shellfish protein phosphatase activity pattern obtained after analysis of tissue from uncontaminated shellfish. Upon feeding on organisms that synthesize DSP toxins, the protein phosphatase activity is reduced considerably, as shown in Figure 1, Lanes 2, 4, and 6. Furthermore, in *A. atter*, both protein phosphatases seem to be equally inhibited; whereas, in *M. chilensis*, the activity of the higher protein phosphatase band is more inhibited than that of the lower band. However, this pattern changes in *V. antiqua*, where the activity of the lower protein phosphatase band is inhibited, and the higher band shows increased activity (Fig. 1, Lane 6) with respect to the uncontaminated control shellfish (Fig. 1, Lane 5).

Dinophysis acuta has been shown to be a DSP toxin producer in Chile (Lembeye et al. 1991, Zaho et al. 1991). Unfortunately, at present, no *D. acuta* cultures are available in Chile or elsewhere. Therefore, to demonstrate that DSP toxins caused the protein phosphatase inhibition observed on the SDS-PAGE, *P. lima* cultures were used to feed *V. antiqua*. In this experiment, two important considerations were taken into account. First, *V. antiqua* was selected, because its inhibitory protein phosphatase pattern is well defined as observed in Figure 1, Lane 6. Second, Quilliam et al. (1996) demonstrated that aged cultures of *P. lima* contain the highest concentrations of okadaic acid and DTX-1 toxins. When the protein phosphatase pattern from shellfish fed with toxic *P. lima* cells are analyzed (Fig. 2, Lanes 2 and 4), it can seen that they are similar to those observed in shellfish naturally contaminated with DSP toxins (Fig. 1, Lane 6). It is also possible to observe that the 140 kDa band has increased protein phosphatase activity when compared to the uncontaminated control band. Furthermore, this increase in protein phosphatase activity resembles the sample naturally contaminated with DSP toxins. The additional 50 kDa protein phosphatase activity band detected is considered as a degradation product during the sample processing of the 70 kDa protein band, because it is also inhibited. However, the possibility exists that this band corresponds to a new protein phosphatase that was not previously detected. Finally, the DTX-1 detection by LC-MS analysis indicates that this DSP toxin inhibited the 70 kDa phosphatase activity in *V. antiqua*. The presence of DTX-1 in Chilean shellfish naturally contaminated with DSP was reported by Zhao et al. (1991).

Okadaic acid is a potent inhibitor of the serine/threonine protein phosphatases PP1A, PP-2A, and PP-2B but has no effect on PP-2C (Cohen 1989). The acetonic shellfish extraction used to detect DSP toxins is performed only on shellfish digestive glands (Japanese Ministry of Health and Welfare 1981), because most DSP toxins are found in this tissue compartment (Lee et al. 1987). However, despite knowing this, there is no available report on how DSP toxins affect endogenous shellfish protein phosphatase, as we describe here. More importantly, a simple analysis of these endogenous protein phosphatase activities could be used as a simple and economical test to demonstrate the presence of DSP toxins in the shellfish.

DSP toxin retention is very low in *Argopecten irradians* as well as in *Mytilus edulis* when fed with *P. lima*, as reported by Bauder et al. (1996) and Pillet et al. (1995), respectively. According to these two groups, low toxicity could be attributed to the possibility that: (1) DSP toxins were not tightly bound to shellfish tissue; (2) *P. lima* cells were evacuated readily with shellfish feces; or (3) DSP toxins follow an "erratic" pattern and are subjected to bio-

conversions. However, based on our results, lower toxicity could also be explained by: (1) a lower number of protein phosphatase molecules acting as DSP binding protein at a given time; or (2) the presence in those shellfishes of a protein phosphatase less sensitive to OA, because OA inhibition varies according to phosphatase isoforms. For example, PP-1, PP-2A, and PP-2B require 20 nM, 0.2nM, and 5 μM of OA, respectively, to be inhibited (Cohen 1989). Therefore, the number of molecules of DSP toxins retained in the shellfish digestive gland may be proportional to the number of DSP-type phosphatase-sensitive molecules available. Thus, the number of OA and DTX-1 molecules bound to phosphatase molecules could correspond to the DSP shellfish toxicity. However, OA and DTX-1 concentration could also depend on such variables as their affinity for the binding protein, the number of newly synthesized protein phosphatase molecules susceptible of being inhibited, the expression of different OA and DTX-1 receptor-like molecules, and the number of phosphatase with or without OA and DTX-1 released during the digestion process by enzymatic or mechanical action or by biotransformations or by protein turnover.

We conclude that DSP toxins inhibit some endogenous shellfish digestive gland protein phosphatases. In addition, the potential exists that changes in the activity patterns of these protein phosphatase molecules could be used to monitor for the presence of DSP toxins in different shellfish species. This approach could become an economic, simple, and sensitive assay capable of detecting the presence of DSP toxins in individual shellfish and avoid troublesome purification steps. Such an assay could be established in most laboratories with minimal investment, allowing the analysis of up to 18 samples within a 4-h period. Finally, this approach could be useful for studies on DSP toxin retention, detoxification of toxic shellfish, and could serve as the target to generate transgenic shellfish resistant or insensitive to toxins causing DSP.

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FIRST REPORT OF DIARRHETIC SHELLFISH TOXINS IN MAGELLANIC FJORDS, SOUTHERN CHILE

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ABSTRACT Diarrhetic shellfish poisoning (DSP) is a gastrointestinal disease caused by polyether toxins produced by dinoflagellates and accumulated in shellfish. Until now, in Chile, harmful algal blooms associated with DSP have been confined to north of 46°00′S. Following a bloom of Dinophysis sp., in Estero Nuñez (53°19′S, 72°30′W) in March 1998, phytoplankton and mussel samples were collected for qualitative and quantitative analysis. Dinophysistoxin-1 (DTX-1), a diarrhetic shellfish toxin, was identified and quantified in extracts of *Mytilus chilensis* Hupe, a chilean native filter bivalve. DTX-1 was measured using precolumn derivatization high-performance liquid chromatography method with fluorometric detection. The presence of DSP toxins was determined by a commercial colorimetric Protein Phosphatase assay (VDM-Test), which proved successful for the rapid screening of shellfish to detect DSP toxins. Okadaic acid was not detected in any mussel samples; the mussel shells showed only the presence of DTX-1, ranging from 65.0–583.8 ng of DTX-1 per gram of mussel digestive gland. This is the first report and quantitative analysis of DSP toxins in the Magellan region and extends 500 miles to the south, the known distribution of DSP toxins in Chilean coasts. The phytoplankton quantitative analysis showed *Dinophysis acuminata* (Clarapéde & Lachmann) as the most probable source of the DSP toxin.

KEY WORDS: *Dinophysis* sp., *Dinophysis acuminata*, DSP toxins, Dinophysistoxin-1 (DTX-1), HPLC toxin profiles, Patagonic fjords, southern Chile

INTRODUCTION

Proliferation of some microalgae in marine waters can contaminate seafood, cause massive fish kills, and alter ecosystems in ways that humans perceive as harmful. These events are generically referred to as harmful algal bloom (HAB). Surveys of affected regions, economic losses, and human poisoning throughout the world demonstrate a dramatic increase in the negative impacts of HABs over the last few decades and that the HAB problem is now widespread (White 1988, Anderson 1989, Smayda 1992, Hallegraeff 1993, Proenca et al. 1997, Lagos 1998).

A broad classification of HABs distinguishes two groups of organisms: the toxin producers, which can contaminate seafood or kill fish even if cell density is low, and high biomass producers, which do not produce toxins but cause deleterious impacts on the environment in other ways, such as through the consumption of oxygen, the production of scums, the reduction in habitat for fish or shellfish, or indiscriminate kills of marine life after reaching dense concentrations. Some HABs have characteristics of both. Although HABs occurred long before human activities began to transform coastal ecosystems, a survey of the affected regions and of the economic losses and human poisonings throughout the world demonstrates that there has been a dramatic increase in the negative impacts of HABs over the last few decades (Hallegraeff 1993, Lagos 1998).

Until now, six important human illnesses associated with microalgae toxins have been described worldwide (Hallegraeff 1993, Yasumoto & Murata 1993, Yasumoto et al. 1995, Falconer 1996). Two of these illnesses are present in Chile: paralytic shellfish poisoning (PSP) and diarrheic shellfish poisoning (DSP), both very well documented (Lembeye et al. 1975, Muñoz et al. 1992, Zao et al. 1993, Uribe 1993, Lagos et al. 1996, Lagos et al. 1998, Compagnon et al. 1998).

The first record of PSP was documented in 1972 in the Magellan region (~48°–56°S), the most austral region of Chile (Guzman et al. 1975, Lembeye et al. 1975), but PSP outbreaks have taken place continuously since 1991 (~42°–55°S.), causing many economic and public health problems (Uribe 1993, Lagos 1998).

Outbreaks associated with DSP have also occurred in the Patagonic fjords since 1970, but until now they have been limited from 46°S north. Historically, the northern part of the Patagonic fjords (more northern than 46°00′S) have been affected with DSP, including localities close to Puerto Montt city (41°27′S, 72°57′W) and some areas in the Aisen Region (~44°–48°S.). Here, some of these outbreaks have been associated with the presence of *Dinophysis acuta* Ehrenberg (Zhao et al. 1993, Lembeye et al. 1993).

Some other species of Dinophysis sp. have been mentioned elsewhere as DSP toxin producers, among them *D. acuminata* Clap. & Lach. and *Phalachroma rotundatum* Clarapéde y Lachmann (Muñoz et al. 1992, Steindinger 1993). Both species, but especially the first one, are very common in the phytoplankton of magellanic fjords (Uribe et al. 1998).

Attempts to evaluate DSP toxins in the Magellan region have been limited to the use of mouse bioassay, mainly in samples from localities near Puerto Eden village (49°20′S) in the northern part of the Magellanic fjords. All of the evaluations were done by a regional monitoring program for preventing poisonings. However, positive results for DSP have been inconclusive for two major reasons: they have not been associated with the presence of toxic or potentially toxic phytoplankton species, and the deaths of mice have been produced without any evidence of gastrointestinal symptoms.

In this paper, we address the presence of DSP toxins in an endemic Chilean filter-feeding bivalve mollusc (*Mytilus chilensis* Hupe) collected in an austral Magellanic fjord during a bloom of Dinophysis sp. This is the first report and quantitative analysis of DSP toxins in this southern remote area in the Magellan region.

MATERIALS AND METHODS

Phytoplankton and Mussel Sampling

Sampling for collecting material was done in Estero Nuñez (53°19′S, 72°30′W) (Fig. 1). A permanent hydrographic station is

maintained close to the sampling site as part of a regional monitoring program for shellfish poisons. The first two visits, in the frame of this program, were scheduled for January 26 and March 23, 1998. At the first visit, we found evidence of water discoloration in the fjord in a patch of . ~½ square mile. A net hauling was transported to the laboratory where the presence of abundant Dinophysis cells was detected.

This finding encouraged a second visit to the fjord, which took place on March 6 in a Chilean Navy Coast Guard vessel. The location where the discoloration was observed was divided into four parcels for sampling (Fig. 1). A set of phytoplankton and shellfish samples was obtained from each parcel. Mussel samples (*M. chilensis*) were collected from an intertidal zone, packed in plastic bags, labeled, and put into the vessel's freezer. Later, the samples were maintained at −20°C until toxin analysis was performed.

Phytoplankton samples for qualitative analysis were taken by vertical hauling of a 20-μm mesh net. A hose displayed in the first 10 m of water column was used to take integral samples for quantitative analysis. Both types of samples were fixed with Lugol's solution. Net samples were observed in normal microscope and quantitative samples in an inverted microscope (Hasle 1978). At least 400 cells per sedimentation chamber were counted.

Mussel Extraction and Analytical High-Performance Liquid Chromatography

Frozen hepatopancreas of mussels (2.0 g) were extracted two times with 3 ml of chilled 80% methanol under mechanical stirring using a tissue tearor (BioHomogenizer M 133/2280; Biospec Products, Inc., Bartlesville, OK). After centrifugation (1,500 × *g* for 5 min), one third of the supernatant (~2.5 ml) was diluted with water to a final concentration of 26.66% methanol. From this dilution, 5 ml were transferred to a C_{18} Sep-Pak cartridge, washed with 5 ml of 50% methanol (discard), and then 5 ml of pure methanol was added to elute the DSP toxins. This eluate was evaporated to dryness under reduced pressure in a Speed Vac Plus (Savant, SC 210A, Farmingdale, NY). The solid residue was treated with a freshly prepared solution of 0.1% ADAM (in 100 μl of acetone and 400 μl of methanol) (Lee et al. 1987). After 1 h at 25°C in the dark, the sample was evaporated to dryness, and the residue was diluted in 200 μl CH_2Cl_2/hexane (1:1) and then applied into a Silica gel cartridge column. The system was washed successively with 5 ml of CH_2Cl_2/hexane (1:1) and 5 ml CH_2Cl_2 and eluted with 5 ml of CH_2Cl_2/methanol (1:1). The last fraction was evaporated to dryness, dissolved in 1 ml methanol, and 10 or 20 μl were analyzed by high-performance liquid chromatography (HPLC). The HPLC conditions were Microsorb-MV C_{18} column (5 μm, 25 cm × 4 mm), mobile phase CH_3CN/methanol/H_2O; 8:1:1 isocratic, flow rate 1 ml/min, and the detection was done by a fluorescence detector (RF-535; Shimadzu), 365 nm (ex.) and 415 nm (em.).

Protein Phosphatase Inhibition Assay

Due to the high sensitivity of the detection, the presence of DSP toxins were also tested using the method based on the enzyme inhibitory activities of Protein Phosphatase 2A (PP2A) (Takai & Mieskes 1991, Rivas et al. 2000). We used two assays, a commercial colorimetric protein phosphatase assay called VDM-Test (TEPUAL S.A., Santiago, Chile) and a second one using the PP2A purified by our laboratory. The PP2A was purified from the filter

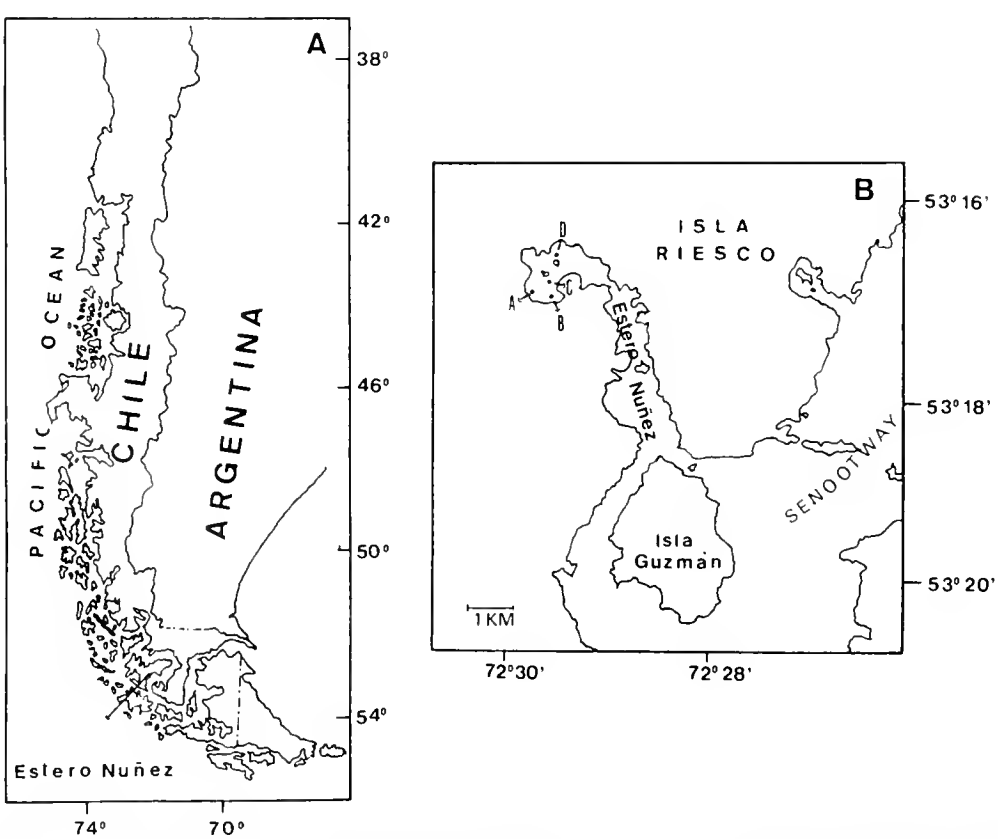

Figure 1. A. Map of Patagonic fjords. B. Sampling location: Sites A, B, C, and D in Estero Nuñez (53°18′00″S, 72°29′00″W).

bivalve *M. chilensis* as described by Rivas et al. (2000). The substrate mixture containing 22 mM *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO), 200 mM Tris/HCl, 20 mM EDTA, 20 mM DTT, and 2 mM $MgCl_2$ (pH 8.31) was mixed with enzyme solution, and the initial rate of liberation of *p*-nitrophenol was measured by recording the change in absorbance at 420 nm in a Beckman spectrophotometer model 25 with a pen recorder (Beckman Instruments, Fullerton, CA). The samples or PP2A inhibitors as positive control were mixed with the enzyme solution 5 min prior to initiating the reaction. Okadaic acid (OA) and Microcystin-LR (both from Sigma Chemical Co) were used as the specific inhibitors for PP2A activities. Complete inhibition was observed with 5 ng of Microcystin-LR or OA. All assays were carried out at 22–24°C. Each determination was done in duplicate. The reaction was stopped with 25 µl of methanol (Rivas et al. 2000).

RESULTS

Phytoplankton Distribution

Sampling on March 6 was made under high cloudiness, which did not permit us to distinguish any water discoloration. A total of 21 diatoms and 23 dinoflagellates, including a cystic form, were observed in the net samples collected (Table 1). The inner station showed the lower species richness (12 spp.), and the others ranged from 23 to 35 spp., most of them dinoflagellates. *D. acuminata* and *P. rotundatum* were detected in these samples.

Cell contour variation of *D. acuminata* seen in the samples showed some deviation from the characteristic oval or elliptical form typically found in Magellanic waters (Fig. 2). Antapex shape varies from rounded (Fig. 2a) to slightly acute (Fig. 2d), having in all cases two or three small protuberances. The light photomicrographs clearly illustrate that the Dinophysis sp., collected in the four stations of monitoring, correspond to *D. acuminata*.

Quantitative samples showed an absolute predominance of *Mesodinium rubrum* Lohmann (Table 1). Dinoflagellate was the second group best represented, and diatoms were present in scarce number (Table 2). From the dinoflagellates, *Gyrodinium lachryma* (Meunier) (Kof. & Swezy) was the most abundant, followed by *Polykrikos schwartzii* (Bütschli) and *D. acuminata*. The concentration of *D. acuminata* ranged from 600–960 cells/l in the four sampled sites. *P. rotundatum* was also present in these samples, although in lower concentrations (Table 1).

Identification and Quantitative Analysis of DSP Toxins

HPLC with fluorescence detection is the most suited approach for the quantitative analysis of DSP toxins. In our study, we used a detection system involving a precolumn derivatization of DSP toxins with a fluorescent chromophore 9-anthryldiazomethane, ADAM (Lee et al. 1987), which allowed us to detect nanogram quantities of DSP toxins. Figure 3A shows a typical chromatogram of DSP reference toxins, such as OA, dinophysistoxin-1 (DTX-1), and the internal standard deoxycholic acid (DEO), showing retention times (Rt) of 9:60, 13:20, and 18:40 min, respectively, for the 9-Anthrylmethyl esters.

Figure 3B shows the chromatographic properties of the extract obtained from the mussel sample collected in site A (see Fig. 1b and Table 2). The elution profile showed a single peak indistinguishable from 9 MA-DTX-1 (Fig. 3A), with a Rt = 13:20 min. All samples extracted and analyzed showed the same elution profile, displaying DTX-1 as the only DSP toxin present in the samples. The amounts of DTX-1 measured by the HPLC analysis of each sample collected in the four sites of Estero Nuñez are showed in Table 2. These values were confirmed by inhibition of

TABLE 1.

Phytoplankton concentration (cells/l) in Estero Nuñez (53°19′00″S, 72°30′00″W).

Species	Sites			
	A	B	C	D
Diatoms				
Bacillaria paradoxa	400	200	200	400
Coscinodiscus sp.	0	0	0	200
Fragilaria sp.	160	0	0	0
Nitzschia longissima	0	0	200	0
Pseudo-nitzschia cf. australis	800	0	0	0
Thalassiosira sp.	40	0	200	0
Dinoflagellates				
Ceratium furca	20	0	20	200
Ceratium pentagonum	200	0	0	200
Dinophysis acuminata	960	680	800	600
Diplopsalis sp.	200	0	0	0
Gymnodinium sp.	400	18,704	1,500	0
Gyrodinium lacryma	14,070	7,484	4,500	0
Phalacroma rotundatum	400	340	240	0
Polykrikos schwarzii	10,553	3,208	500	2,000
Protoperidinium conicum	0	200	0	0
Protoperidinium sp.	0	200	0	0
Silicoflagellates				
Dyctiocha speculum	0	0	200	0
Subtotal	28,203	31,016	8,360	3,600
Mesodinium rubrum	159,795	36,353	133,000	135,200

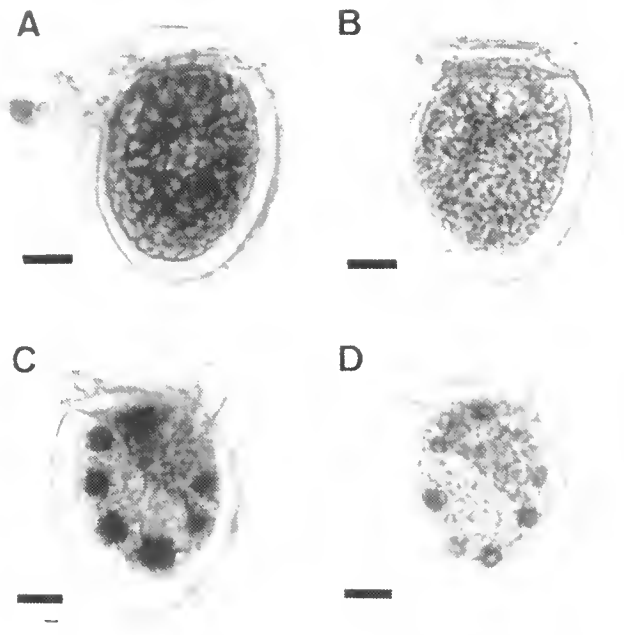

Figure 2. Contour variation of *Dinophysis acuminata* (Clarapéte & Lachmann) found in Estero Nuñez.

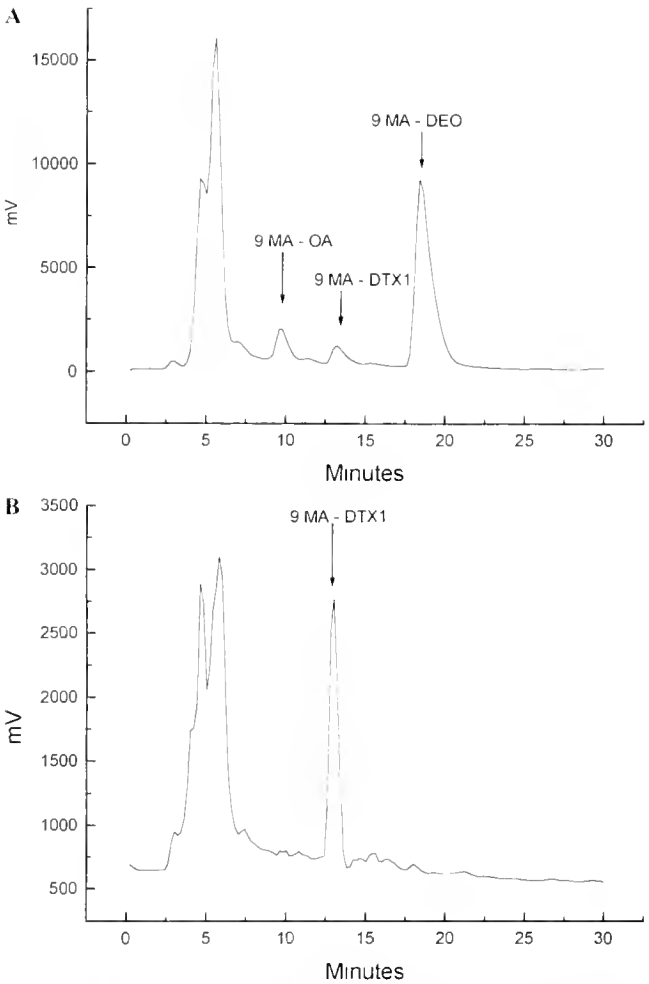

Figure 3. A. Fluorometric high-performance liquid chromatography (HPLC) chromatogram of diarrhetic shellfish toxin standards derivatized with ADAM: 9MA-OA (9-anthrylmethyl Okadaico), 9MA-DTX1 (9-anthrylmethyl Dinophysistoxin 1), and 9MA-DEO (9-anthrylmethyl Deoxycholic). All esters are from constituents that have carboxylic acid. B. Fluorometric HPLC chromatogram of diarrhetic shellfish toxin present in mussel extracts derivatized with ADAM: (9-anthrylmethyl Dinophysistoxin 1).

PP2A assays. The extracts of mussel samples collected from sites A, B, and C showed inhibition over PP2A, the amounts of DSP toxin calculated from the inhibition curve using the mussel extracts were the same measured by HPLC (Table 2).

DISCUSSION

From the low phytoplankton concentrations and the absence of water discoloration it is possible to infer that the sampling done in March 6 was made in a period after a bloom. Samples came from the head of a small, enclosed fjord that receives fresh water discharge from two creeks, so the water column stability is a typical situation in this area. During the monitoring visit, done on January 26 and March 23, the temperature and salinity were recorded from a monitoring station located approximately 2 miles from the sites where samples were collected. These data showed a low density in surface water with an average temperature of 11.18°C and a mean salinity of 28.78 psu in January. During the monitoring visit of March, these parameters were 9.14°C and 29.49 psu, respectively. At both sampling visits a pycnocline at approximately 5 m depth was observed. According to the literature, such stability is a condition that permits planktonic dinoflagellates and ciliates to reach high densities (Margalef et al. 1979, Holligan 1985).

D. acuminata is one of the seven species of Dinophysis sp. that have been confirmed to produce OA and/or DTX-1 (the other six species are *D. fortii* Pavillard, *D. acuta*, *D. norvegica clarapede* & *Lachmann*, *D. mitra* (Schutt) Abé, *D. tripos* Gourret, and *Ph. rotundatum*; Lee et al. 1989). The quantitative analysis of phytoplankton samples collected in the Estero Nuñez showed two possible sources of DSP toxins: *D. acuminata* and *P. rotundatum*. *P. rotundatum* is the most likely source because it showed the

TABLE 2.

Amount of DTX-1 detected in mussel extracts collected in the four sites of Estero Nuñez.

Samples	Location	Date	OA (ng/g)	DTX-1 (ng/g)
Mytilus chilensis Hupe	Estero Nuñez (site A; see map, Fig. 1B)	March 6	ND	583.8
Mytilus chilensis Hupe	Estero Nuñez (site B; see map, Fig. 1B)	March 6	ND	117.5
Mytilus chilensis Hupe	Estero Nuñez (site C, see map, Fig. 1B)	March 6	ND	65.0
Mytilus chilensis Hupe	Estero Nuñez (site D, see map, Fig. 1B)	March 6	ND	ND

DTX-1 = dinophysistoxin-1; OA = okadaic acid; ND = none detected.

higher cell density. *D. acuminata* is a common species in the phytoplankton of the Magellanic fjord waters (Uribe et al. 1998). The variation of the contour found this time in the *D. acuminata* cells is not common in the Magellanic fjords, but it adjusts quite well in the range presented by Balech (1976) in a study of Norwegian Dinophysis.

Estero Nuñez has the typical geomorphology of small Magellanic fjords, with the water column stability as the most common hydrographic feature. Consequently, small toxic blooms could be frequent along the region and probably are being overlooked by monitoring programs because sampling stations are located far from the fjord heads. In fact, mussels gathered in the head of Estero Nuñez caused eight human cases of PSP in April 1989 in this region (Uribe 1989, Lagos 1998). A similar situation occurred with mussels collected in the head of Bahía Nash, in March 1991, where there were 95 human cases of PSP and two deaths (Uribe 1992, Lagos 1998).

Historically in our country, HABs associated with DSP have been confined to Patagonic fjords north of 46° S. Now, this finding extends the known DSP toxins distribution approximately 500 miles further to the South of Chile in the Austral Magellanic fjords.

The frequent presence of Dinophysis sp. in Magellanic fjord waters, now associated with DSP toxins, represents a latent threat to the public health, which demonstrates the need to develop a more thorough evaluation for the presence of DSP toxins and its primary source in many small fjords along the Magellanic region. This is especially important after the demonstration that DTX-1 causes severe injuries on the intestinal mucosa (Terao et al. 1986). Furthermore, another important aspect of OA and DTX-1 is their potent tumor-promoting activity (Suganuma et al. 1988), so attention should be paid to the continuous uptake of subacute levels of DSP toxins through seafood.

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AMNESIC SHELLFISH POISONING IN THE KING SCALLOP, *PECTEN MAXIMUS*, FROM THE WEST COAST OF SCOTLAND

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ABSTRACT The king scallop, *Pecten maximus*, is a valuable economic resource in the UK. The industry relies on supplying premium "roe-on" processed scallops to the continental market. In July 1999, king scallops harboring the amnesic shellfish poisoning (ASP) toxin, domoic acid (DA), in gonadal tissue at levels above the regulatory limit (20 μg DA g^{-1}) were detected across a wide area of northern and western Scotland. In response, a survey of the southern extent of the closed harvest areas was initiated to describe variability of ASP toxin levels over varying spatial scales (<5 m to >5 km); determine the anatomical distribution of the toxin, and identify, isolate, and culture causative *Pseudo-nitzschia* species. Toxin analysis was conducted using a liquid chromatography-tandem mass spectroscopy (LC-MS/MS) procedure. The DA content of tissues followed the predictable rank order: all other tissue → gonad → adductor. The toxin levels within all other tissue (95% CI = 580–760 μg DA g^{-1}, n = 170) consistently accounted for 99% of the total individual toxin burden. DA levels in the gonad (95% CI = 8.2–11.0 μg DA g^{-1}, n = 170) were an order of magnitude below levels in all other tissue and contributed to less than 0.5% of the total individual toxin burden, although levels above the regulatory limit were detected in individual gonad samples. Adductor muscle tissue contained the lowest concentrations of DA (95% CI = 0.38–0.82 μg DA g^{-1}, n = 170), and was typically within two to three orders of magnitude below levels in all other tissue. None of the scallops examined had DA toxicities in adductor muscle tissue exceeding the regulatory limit. Toxin variability among individuals and sites was high (range of coefficients of variation (CV) in all other tissue = 29%–120% and gonadal = 45%–85%). The results do give an indication of the scale on which microhabitat differences may influence ASP toxicity in *P. maximus* populations, because significant differences were found in all other and gonadal tissue toxin levels between groups of individuals only 25-m apart. In total, seven species of *Pseudo-nitzschia* were identified from west coast waters. A suspected causative species, *P. australis*, was found to produce high levels of DA, in culture. The high individual variation in toxicities and the occurrence of DA in the gonad at levels above the regulatory limit clearly demonstrate the complexity of managing the king scallop fishery during ASP events.

KEY WORDS: amnesic shellfish poisoning, domoic acid, *Pseudo-nitzschia*, *Pecten maximus*, scallop fishery

INTRODUCTION

Marine algal toxins comprise a diverse group of biologically active compounds with high acute toxicities in humans (Shumway & Cembella 1993). Scallops, opportunistic filter feeders exploiting both pelagic and benthic microorganisms as food sources, are liable to the accumulation and concentration of phycotoxins from toxic algal species present in the water column (Shumway et al. 1987, Bricelj & Shumway 1991). The risk of human illness as a result of toxic scallop consumption poses a significant threat to both public health and shellfish industries (Shumway & Cembella 1993).

Amnesic shellfish poisoning (ASP), a relatively new type of seafood toxicity, was first described from Prince Edward Island, Canada, in 1987 (Bates et al. 1989). Over 100 people who consumed mussels contaminated with a naturally occurring neuroexcitatory toxin, domoic acid (DA), experienced gastroenteritis and neurological symptoms (Wright et al. 1989, Todd 1993). In this first episode, the source of the domoic acid was identified as the pennate diatom, *Pseudo-nitzschia pugens* f. *multiseries*, which was ingested and accumulated by the mussels during normal filter feeding (Bates et al. 1989). Global awareness of ASP has since been raised, and, to date, ASP toxin-producing species of *Pseudo-nitzschia* have now been reported from the gulf of Mexico region, North America, Canada, Europe, Australia, Japan, and New Zealand (Hallegraeff 1995). From laboratory studies, it is now clear that several species of *Pseudo-nitzschia*, and two species from separate genera (*Amphora* and *Nitzschia*), are capable of producing DA, but the levels of production are highly variable (Kotaki et al. 2000, Bates 2000, Lundholm & Moestrup 2000).

The king scallop, *Pecten maximus*, is a valuable economic resource in the UK. The UK scallop industry is principally a wild fishery exploited by scallop dredgers, which account for an estimated 97% of UK landings. However, small quantities are landed by divers, and in Scotland, there is an emergent aquaculture industry. The industry is largely reliant on supplying premium "roe-on" processed scallops to the continental market. In 1998, 9,700 tons of *P. maximus* were landed in Scotland, with a first sale value of £15.5 million, equating to approximately 25% of all EU scallop landings (Denton 1999). An estimated 95% of the king scallops are processed as meat and roe product, of which 60% is distributed as premium chilled product and 40% frozen. Dive collected and farmed scallops are sold whole to a smaller, yet higher value market for live shellfish.

The incorporation of systematic ASP/domoic acid testing of shellfish into the Food Standards Agency (FSA) Scottish waters surveillance program was initiated early in 1999. By July 1999, *P. maximus* harboring DA in the gonad at levels above the internationally accepted closure limit (20 μg DA g^{-1}) were detected across a wide area of northern and western Scotland. This prompted a widespread closure of the king scallop fishery, which persisted in excess of 9 months, resulting in financial hardship for scallop dredging, diving, and cultivation industries. The nature of

the ubiquitous and prolonged high toxicity seemed to be confined to the king scallop, because only sporadic, short-term toxicities were noted in the queen scallop, *Aquipecten opercularis*, and negligible levels detected in other shellfish (FSA pers. comm., 1999). The direct cost to the industry to date has been estimated at £10 million, and the loss of skilled processing staff and disruption of established supply routes to continental markets led to serious concern for its survival (Denton 1999). The restriction on all scallop landings provoked controversy, stimulating much media interest. To date, there has been no documented history of human illness caused by ASP in the UK.

In response, an opportunistic survey of the southern region of the closed harvest areas was initiated to provide fundamental information on the ASP incident. The objectives were to: describe ASP toxin variability among individual and neighboring scallop populations over varying spatial scales (<5 m to >5 km); determine the anatomical distribution of ASP toxin within scallop body parts: adductor muscle and gonad, and all other tissue (digestive gland, mantle, gill); assess any influence of size, age, and depth on scallop toxicity levels; and isolate, culture, and identify causative *Pseudo-nitzschia* species. The data collected provide basic information to assist with the development of rational management strategies to continue to protect public health while minimize the economic constraints of future ASP events.

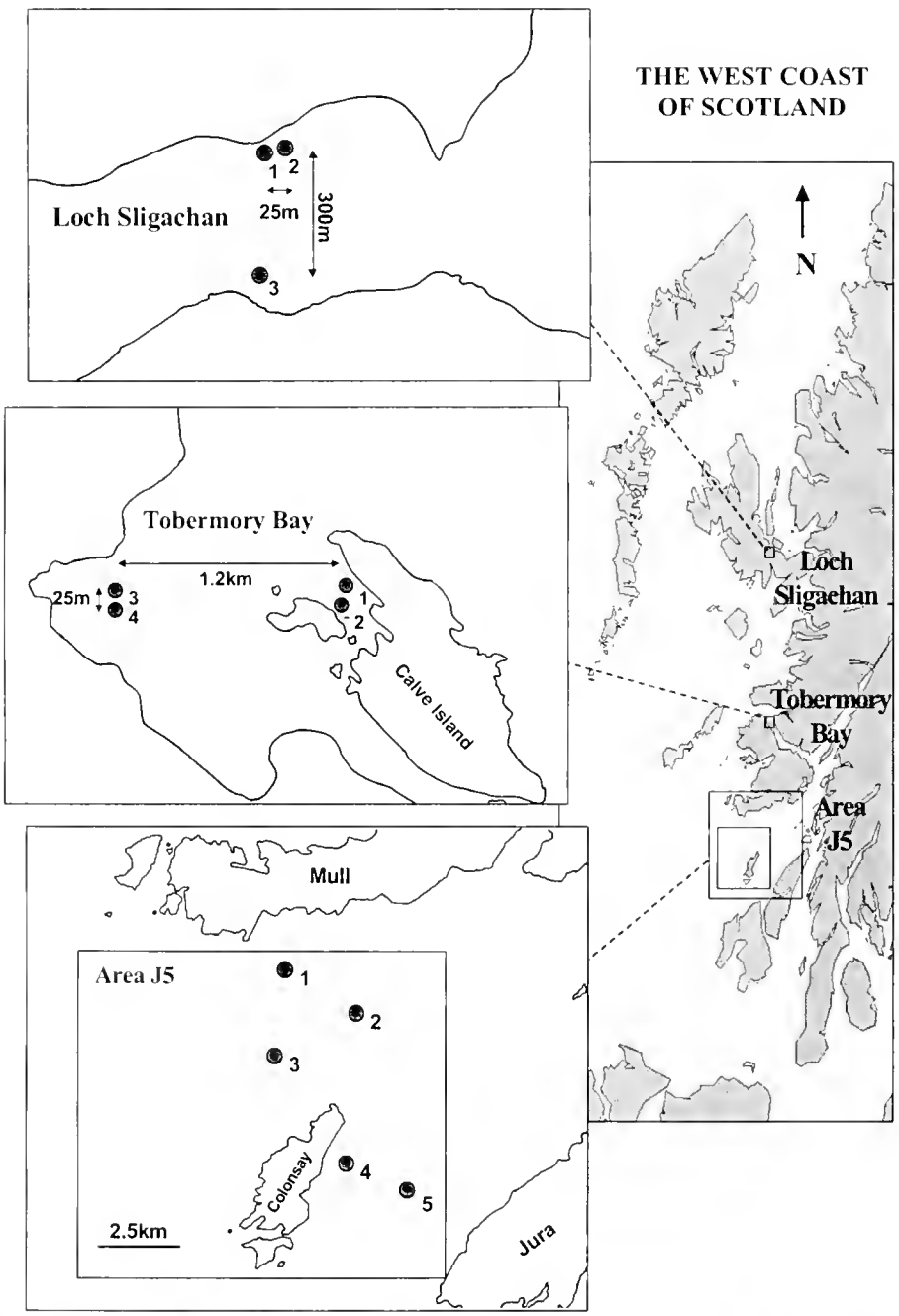

Figure 1. Map of the west coast of Scotland showing the study sampling locations and subsites within locations.

TABLE 1.

Overall mean and standard error (SE) DA levels (μg DA g^{-1} wet weight) in the adductor, gonad, all other tissue and total tissue of *P. maximus* collected from the three locations.

Tissue	*n*	Mean	SE	Minimum	Maximum	CV (%)	95% CI	no. >20 μg g^{-1}	no. >1000 μg g^{-1}
Adductor	170	0.60	0.114	0.011	15.42	247	0.38–0.82	0	—
Gonad	170	9.58	0.722	0.131	75.5	98	8.16–11.01	19	—
All other	170	669	45.7	0.2	3689	89	580–760	169	10
Total	170	295	19.3	0.9	1569	85	256–333	168	8

Ninety-five percent confidence intervals (CI), minimum and maximum levels of DA obtained, coefficient of variation (CV %) and number of individuals with toxin burdens >20 and >1000 μg DA g^{-1} are given.

MATERIALS AND METHODS

Sampling

In December 1999, 10 specimens of adult *P. maximus* (shell height >90 mm) were collected by SCUBA at three subsites (total of 30 individuals) within Loch Sligachan (57°15′5′ N 06°15′5′ W) and at four subsites (total of 40 individuals) within Tobermory Bay (56°35′5′ N 06°05′5′ W) (Fig. 1). To provide an assessment of ASP toxin levels among scallop populations on a larger spatial scale, in context with the current monitoring program; a third sampling location, the scallop fishing box-Area J5, was included in the sampling regime (Fig. 1). Twenty adult scallops (shell height >90 mm) were selected at random from the landings dredged from each of five subsites (total of 100 individuals). These locations were routinely used for the monitoring of ASP toxin under the Food Standards Agency Program and were chosen as a result of previously consistent high DA levels (above the 20 μg DA g^{-1} statutory level) within scallop gonad. Upon collection, all scallops were individually sealed in zip-lock polythene bags, placed in cool boxes, and transported to the Scottish Association for Marine Science (SAMS) Laboratory within 6–12 h, for immediate dissection.

The age of each scallop was estimated by enumerating shell growth bands and shell length and breadth (Mason 1983) measured to the nearest 0.1 mm. The scallops were dissected into the body components: adductor muscle, gonad, and all other tissue (digestive gland, mantle, and gills). Special care was taken to avoid artifactual contamination from adjacent tissues, by careful dissection, washing, and drying of individual body components. The digestive material of the intestinal loop within the gonad was physically removed. All body components were weighed to the nearest 0.001 g, sealed separately in zip-lock polythene bags, and frozen at −20°C before DA extraction (Quilliam et al. 1989).

DA Extraction and Quantification in Scallop Tissue

Tissues were homogenized in a blender (3 min), which was cleaned and rinsed with methanol and then distilled water, between each sample. Four grams of tissue homogenate was rehomogenized (4 min) with 10 mL of 100% methanol, centrifuged (10 min at 5,000 rpm), and a 5-mL subsample of the resulting supernatant filtered through a disposable 45-μm filter membrane. The extract was stored at −20°C before DA detection and quantification.

The extracts were evaporated to dryness using vacuum centrifugation and resolubilized in 50/50 methanol and water before triplicate analysis. The samples were analyzed on a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system consisting of an Agilent Model 1100 high-performance liquid chromatography (HPLC) system, coupled to either a SCIEX API-III triple quadrupole or a Finnigan LCQ ion trap mass spectrometer. The chromatography was performed on a C18 reversed phase column with a 0.2 mL/min flow of a 1–95% gradient of methanol:

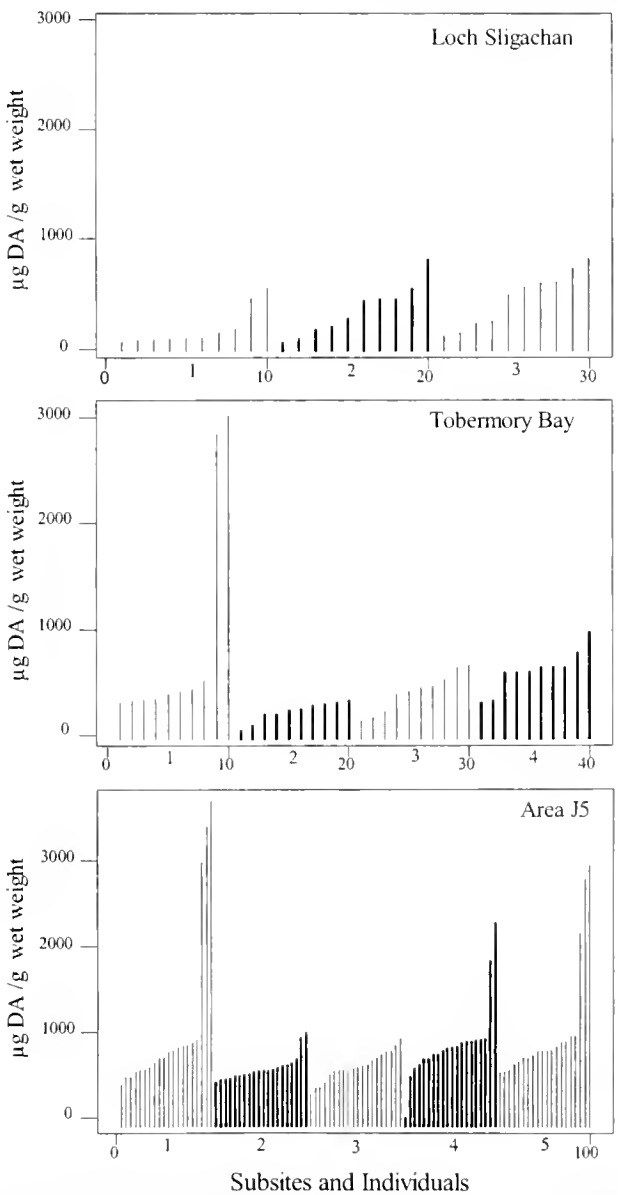

Figure 2. DA toxicity levels (μg DA g^{-1} wet weight) in all other tissue of *P. maximus* collected from subsites within locations.

water. All solvents had 0.1% trifluoroacetic acid added. A portion of the effluent from the HPLC system was directed into the electrospray ionization source of the mass spectrometer via a flow splitter. The mass spectrometer was operated in positive mode with [M+H]+ ions (−312 m/z) being isolated in a first stage of MS analysis. The isolated ions were subjected to "collision- induced dissociation" reaction conditions, which are expected to stimulate the fragmentation of the [M+H]+ ions into characteristic product ions. All quantitations were based on the integrated chromatographic intensity areas of one of the fragment ions (at 267 m/z), and the appearance of other characteristic ions was used as confirmatory evidence for the DA ion's identity (Scholin et al. 2000).

Phytoplankton Sampling and Isolation

During August and October, plankton tows were taken from Orkney, Dunstaffnage, and Jura, to record *Pseudo-nitzschia* spp. presence. In December, at each subsite within Loch Sligachan and Tobermory Bay, surface plankton trawls, quantitative water samples (NIO bottle, at 5 m), cores (water/sediment interface samples), and benthic sediment samples were taken to examine the vertical distribution of *Pseudo-nitzschia* spp. present. At the subsites within Area J5, plankton trawls and quantitative water samples with (NIO bottle, at 5 m) were performed. Four replicates of each sample were obtained from each site; of which two were preserved with Lugol's iodine for cell counts, and two were enriched by addition of f/2 + Si growth medium. Samples were examined for actively growing chains of *Pseudo-nitzschia* cells and candidate chains of cells isolated by micro-pipette and repeated washing in sterile f/2 growth medium. Individual chains of cells were incubated with f/2 + Si growth medium (Guillard & Ryther 1962) at 15°C under an approximate light intensity of 50–80 μmol PAR m^{-2} s^{-1} (12:12 light/dark cycle). Stock cultures were grown for three weeks (stationary phase) before cell harvest by gentle centrifugation (1,500 rpm), followed by removal of excess growth medium and resuspension in sterile growth medium. Samples of cell pellets and supernatant were immediately placed on ice before DA analysis.

DA Extraction and Quantification in Pseudo-nitzschia *spp.*

Cell pellets were subjected to ultrasonication (10 min) in 50/50 methanol and water, before filtration (Whatman filter, 0.2 μm). The supernatant samples were directly filtered using the same filter size. The crude extract or filtrate from the supernatant was analyzed using HPLC coupled to a diode-array detector (10 μL injected). The system (Thermoquest) comprised a solvent reservoir and degasser, P4000 pump, AS3000 autosampler, and UV 6000 diode-array detector. The HPLC column was a VYDAC 201TP54 (250 × 4.6 mm, 5 μm) with a VYDAC guard column 201GK54T (10 × 4 mm, 5 μm). The mobile phase was 0.1% trifluoracetic acid in 10% aqueous acetonitrile, at 1.5 mL/min flow rate. The column temperature was kept at 40°C. Wavelengths monitored ranged from 200–360 nm, and spectral confirmation was obtained by comparison of sample spectra to those from the certified reference standard DACS-1C. Quantitation was carried out at a wavelength of 242 nm.

RESULTS

Anatomical Distribution

Despite the considerable variation in toxin levels within each body compartment, DA loading of the tissues followed a predictable rank order: all other → gonad → adductor. The toxin levels within all other tissue consistently accounted for 99% of the total DA burden. A small proportion of individuals had DA levels in all other tissue (>1,000–3,690 μg DA g^{-1}) an order of magnitude greater than mean levels (669 ± 45.7 μg g^{-1}). Mean DA levels (μg

TABLE 2.

Mean and standard error (SE) of DA levels (μg DA g^{-1} wet weight) in all other tissue of *P. maximus* at each sample location and subsite.

Location and subsite	*n*	Mean	SE	Minimum	Maximum	CV (%)	95% CI	no. >1000 μg g^{-1}
Loch Sligachan	30	330[a]	44.2	58.6	820	73.4	240–420	——
Tobermory Bay	40	537[a]	93.5	45.2	3023	110	348–726	2
Area J5	100	824[b]	62.0	0.2	3689	75.3	701–947	8
Loch Sligachan								
1	10	183[a]	54.7	62.5	549	94.6	59–306	——
2	10	352[ab]	73.9	58.6	814	66.2	185–520	——
3	10	455[b]	79.3	123	820	55.1	275–634	——
Tobermory Bay								
1	10	895[a]	341	307	3023	120	123–1670	2
2	10	228[b]	29.6	45.2	331	41.5	296–1601	——
3	10	407[ab]	57.8	137	663	45.0	276–538	——
4	10	618[a]	61.1	318	984	31.2	479–756	——
Area J5								
1	20	1076[ab]	224	379	3689	93.0	607–1545	3
2	20	581[a]	33.5	409	998	25.8	510.6–651	——
3	20	586[a]	37.7	275	915	28.8	507–665	——
4	20	861[b]	104	0.2	2270	53.8	645–1078	2
5	20	1016[b]	159	525	2928	70.1	683–1350	3

Means from the same location with different superscripts are significantly different ($P > 0.05$, Kruskall–Wallis and Dunn's method). Ninety-five percent confidence intervals (CI), minimum and maximum levels, coefficient of variation (CV %) and number of individuals with toxinburdens >1000 μg DA g^{-1} are given.

g^{-1}) in gonad tissue were an order of magnitude below levels in all other tissue, and, on average, contributed to less than 0.5% of the total individual toxin burden. DA levels above the statutory 20 μg DA g^{-1} safety level in gonads were detected in 22% of the scallops examined, although these values were not encompassed within the 95% confidence limits (8.16–11.01 μg DA g^{-1}, $n = 170$). Adductor muscle contained the lowest concentrations of DA and was typically two to three orders of magnitude below levels in all other tissue while accounting for only 0.17% (mean) of the total individual toxin burden. Although the CV (247%) of individual adductor muscle DA levels was observed to be considerably greater than all other and gonad tissues (98 and 89%, respectively), none of the scallops examined, had adductor muscle toxicities that exceeded the statutory limit, and 95% of the samples had levels below 1.9 μg g^{-1}.

A weak, positive correlation was observed between $\log_{10}$ all other tissue toxicity and $\log_{10}$ gonadal toxicity ($r = 0.303$, $P < 0.001$, $df = 169$); whereas, no correlation could be found between DA concentrations in all other and adductor muscle tissue. No significant correlation could be made between DA toxicity in the three body compartments and scallop age and size parameters.

Spatial Distribution

At all sites, a large variation in all other tissue toxin levels between individuals was observed (Fig. 2), indicated by the CV values (Table 2). However, significant differences in all other tissue toxin levels among locations were distinguished. Scallops from Area J5 had significantly greater levels of DA in all other tissue than individuals from Tobermory Bay and Loch Sligachan; whereas, no significant differences in toxin levels were found between scallops from Tobermory Bay and Loch Sligachan.

Significant differences in toxicities between individuals from different subsites within the same location were observed at all the three locations. Within Tobermory Bay, all other tissue DA toxin levels differed significantly between neighboring scallop populations 25 and 1,200 m apart (subsite 2 DA levels were significantly lower than 1 and 4). In scallops from Loch Sligachan, all other tissue DA toxin levels differed between neighboring scallop populations 300 m apart (subsite 3 DA levels were significantly higher than 1). Within the scallop fishing box, Area J5, all other tissue DA toxin levels were significantly higher in scallops from subsites 4 and 5 than 2 and 3, collected 8–12 km apart. Scallops with high all other tissue toxin burdens (>1,000 μg g^{-1}) were not evenly distributed among locations or subsites. The significance of the results remained unchanged, regardless of the removal of individuals with high toxicities from the dataset.

At all sites, a large variation in gonadal toxin levels between individuals was observed (Fig. 2), indicated by CV (Table 3). Despite the wide variation, gonadal toxicity among locations followed the same pattern of the toxicity as all other tissue, because scallops from Area J5 had significantly greater levels of DA in the gonad than individuals from Tobermory Bay and Loch Sligachan. Similarly, no significant differences in gonad toxin levels were found between scallops from Tobermory Bay and Loch Sligachan.

Significant differences in DA levels between subsites of the same site were observed at all threesites sampled. In Tobermory Bay, gonad DA toxin levels differed between neighboring scallop populations 25-and 1,200-m apart (subsite 2 and 3 DA levels were significantly lower than 1). In Loch Sligachan, gonadal DA toxin levels differed between neighboring scallop populations 300 m

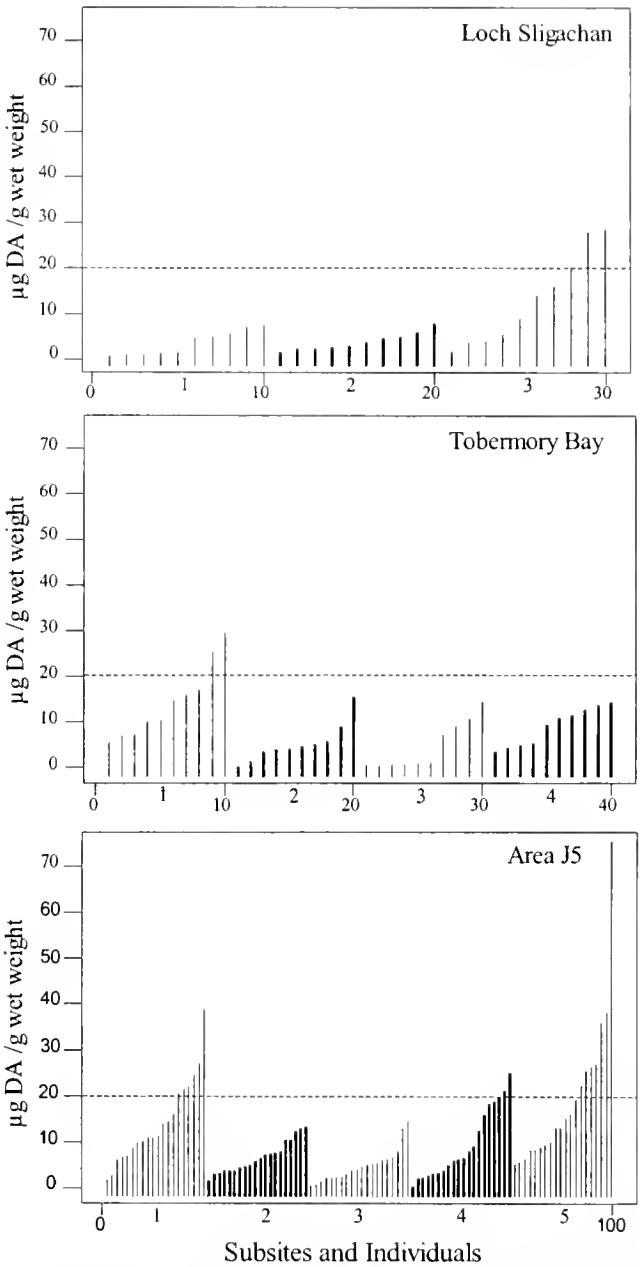

Figure 3. DA toxicity levels (μg g^{-1} wet weight) in the gonad of *P. maximus* collected from subsites within locations. Line = statutory limit (20 μg DA g^{-1} of tissue).

apart (subsite 1 DA levels were significantly higher than 3). Within Area J5, gonadal DA toxin levels were significantly higher at subsite 5 than at 2, 3, and 4; whereas, gonad toxicity in subsite 1 was greater than that in 3. At each location, scallops with gonadal toxicities exceeding the 20 μg DA g^{-1} limit were encountered. However, the frequency of these individuals was not homogenous among the subsites, ranging from 0 out of 20 individuals (Area J5, subsites 2 and 3) to 7 out of 20 (Area J5, subsite 5).

Scallops from Tobermory Bay had significantly lower levels of DA in the adductor muscle than individuals from Loch Sligachan and Area J5. Levels of toxicity among locations did not correspond to the pattern of toxicity seen in all other and gonad tissue. No significant differences in adductor muscle toxin levels were found

TABLE 3.

Mean and standard error (SE) of DA levels (μg DA g^{-1} wet weight) in the gonad of *P. maximus* at each sample location and subsite.

Location and subsite	*n*	Mean	SE	Minimum	Maximum	CV (%)	95% CI	no. >20 μg g^{-1}
Loch Sligachan	30	6.82[a]	1.35	0.77	28.62	108	4.06–9.58	3
Tobermory Bay	40	8.18[a]	1.06	0.13	29.49	81.9	6.04–10.32	2
Area J5	100	10.97[b]	1.06	0.36	75.47	96.8	8.87–13.08	15
Loch Sligachan								
1	10	3.52[a]	0.847	0.775	7.517	76.1	1.6–5.4	——
2	10	3.93[a]	0.622	1.513	7.939	50.1	2.5–5.3	——
3	10	13.02[b]	3.17	1.72	28.62	77.0	59–20.2	3
Tobermory Bay								
1	10	14.14[b]	2.54	5.48	29.49	56.8	8.4–19.9	2
2	10	5.21[a]	1.38	0.13	15.55	83.5	2.1–8.3	——
3	10	4.41[a]	1.66	0.32	14.18	119	0.7–8.2	——
4	10	8.95[ab]	1.32	3.33	14.28	46.7	6.0–11.9	——
Area J5								
1	20	14.17[ac]	2.05	1.80	38.84	64.9	9.9–18.5	6
2	20	6.77[ab]	0.792	1.593	13.28	52.3	5.1–8.4	——
3	20	4.86[b]	0.823	0.594	14.59	75.7	3.1–6.6	——
4	20	9.57[ab]	1.71	0.36	25.15	79.7	6.0–13.1	2
5	20	19.50[c]	3.68	5.21	75.47	84.5	11.8–27.2	7

Means from the same location with different superscripts are significantly different ($P > 0.05$, Kruskall–Wallis and Dunn's method). Ninety-five percent confidence intervals (CI), minimum and maximum levels of DA obtained, coefficient of variation (CV %) and number of individuals with gonadal toxin burdens >20 μg DA g^{-1} statutory limit are given.

between scallops from Loch Sligachan and Area J5. At all sites, an exceptionally large individual variation in adductor muscle toxin levels was observed (Fig. 4), indicated by the CV (Table 4).

Within Tobermory Bay, adductor muscle DA toxin levels differed between neighboring scallop populations 25- and 1,200-m apart (subsite 3 DA levels were significantly lower than 1). In Loch Sligachan, no significant differences in adductor muscle toxin levels were observed between subsites. In Area J5, adductor muscle toxin levels were significantly higher in scallops in subsite 5 than in 2 and, 3, and toxicity in subsite 1 was greater than in 2. Again, the significance of the results remained unchanged, regardless of the removal of individuals with a comparatively high toxin loading.

Pseudo-nitzschia *spp. Abundance and DA Production*

The August to October plankton tows samples showed several potentially toxic *Pseudo-nitzschia* species were present. At the peak of the blooms, *P. australis* was the dominant species followed by *P. pungens*. Several other species were present as minor components: *P. multiseries*, *P. seriata*, *P. delicatissima*, *P. fraudulenta*, and *P. pseudodelicatissima*. The blooms were observed to subside during October 1999, with low levels of *P. delicatissima* persisting through to December 1999. At the time of scallop collection (December 1999) *Pseudo-nitzschia spp.* cell numbers were exceptionally low throughout the water column (<1 cell/mL) at the sites sampled (water temperature range 7–9.5°C). These cell concentrations are well below that usually associated with reported ASP events. Examination of surface sediments at Loch Sligachan and Tobermory Bay also failed to detect significant quantities of living or dormant cells *Pseudo-nitzschia spp.*

Three *Pseudo-nitzschia* cultures were established from samples collected in the August 1999 blooms, two strains of *P. australis*, and one of *P. pungens*. Stationary growth-phase cultures of both *P. australis* strains produced detectable levels of DA in intracellular and extracellular fractions (Table 5). However, the presence of DA could not be detected in the *P. pungens* cultures (detection limit = 0.1 μg mL^{-1} in cell/supernatant extracts). In both *P. australis* cultures, total DA was partitioned with approximately one- third being intracellular and two-thirds present in the growth medium.

DISCUSSION

The trend in body component toxicity of *P. maximus* as a proportion of total scallop toxin burden (all other tissue → gonad → adductor), is in agreement with previous studies of DA in *P. maximus* (Arevalo et al. 1998), DA in *Placopecten magellanicus* (Douglas et al. 1996), and paralytic shellfish poisoning (PSP) in *P. magellanicus* (Cembella et al. 1994). In the current study, 99.4% of individuals had levels of DA in all other visceral tissue over the statutory 20 μg DA g^{-1} limit. The maximum DA concentration in all other tissue (3,689 μg DA g^{-1}), recorded in this study was approximately 180 times the regulatory limit (20 μg DA g^{-1}) and is among the highest levels recorded in bivalves. Arevalo et al. (1998) found the highest levels of DA in the hepatopancreas in *Pecten maximus* (maximum = 2,083 μg DA g^{-1}). Similar high levels (approximately 3,000–4,000 μg DA g^{-1}) were found in the digestive gland of *Placopecten magellanicus* (cited in Douglas et al. 1996). Thus, the levels of DA found in all other tissue in the present study are consistent with previous findings, confirming that DA is predominantly sequestered within the digestive gland in *P. maximus*.

Toxin levels of gonadal tissue were generally lower than the statutory 20 μg DA g^{-1} limit; however, toxicities above this level were encountered. Adductor muscle toxicity contributed negligible amounts to the total body burden, and levels never exceeded the statutory limit, even when toxin levels were extremely high in all other tissue. The occurrence of PSP toxins in adductor muscle is

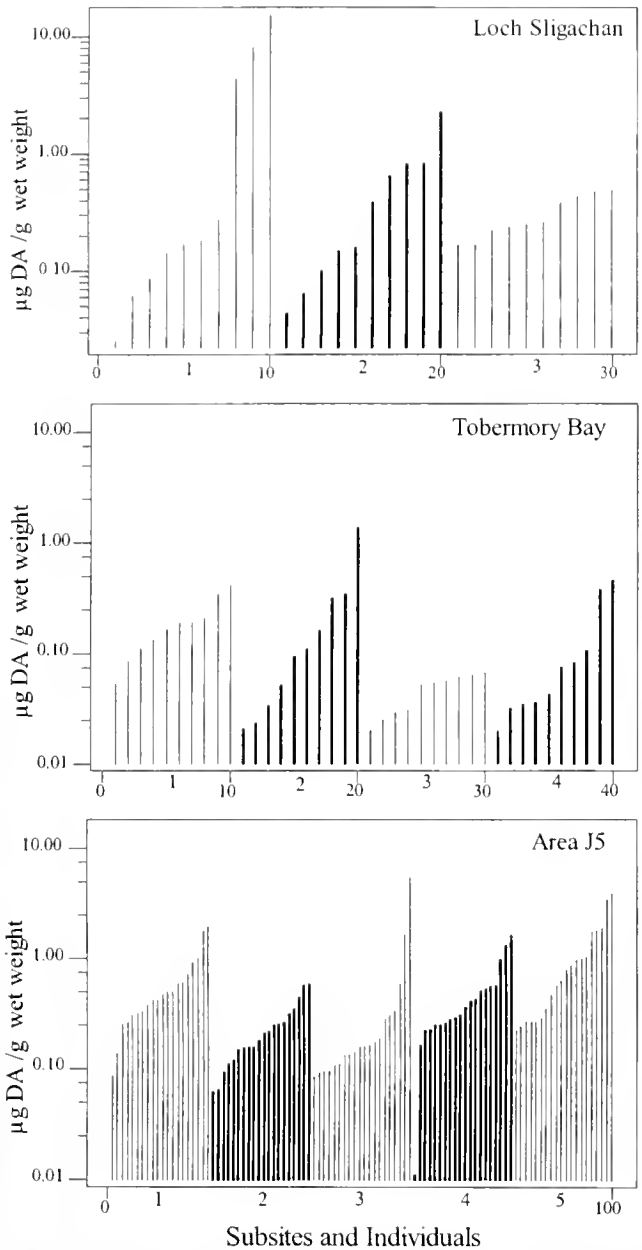

Figure 4. DA toxicity levels (µg DA g^{-1} wet weight) in the adductor muscle of *P. maximus* collected from subsites within locations, plotted against a $\log_{10}$ scale.

rare; and in the scallops *P. magellanicus*, *Patinopecten yessoeniss*, and *Crassodoma gigantea*, adductor muscle PSP toxicity is always at least 10-fold less than in corresponding digestive tissue (cited in Shumway & Cembella 1993). Bricelj and Shumway (1998) report that, during PSP events, such tissues involved in locomotion as the muscular foot, adductor muscle, and pallial muscles, invariably attain toxicities two to three orders of magnitude below those in the viscera, and contain a minimal proportion, typically < 1% of the total toxin loading, despite their relatively large mass in some species. However, Pacific razor clams, *Siliqua patula*, are reported to sequester DA principally within muscular tissue (Drum et al. 1993).

Confirmation of DA production by *Pseudo-nitzschia australis* and its dominance during the blooms indicate this species was one of the primary sources of scallop ASP contamination in 1999. Although a co-dominant species, *Pseudo-nitzschia pungens*, did not produce detectable concentrations of DA in culture, it is possible that other *Pseudo-nitzschia* species, with known DA production capabilities and present as minor components, may have contributed to the ASP event (e.g., *P. seriata*, *P. fraudulenta*, or *P. pseudodelicatissima*). The dominance of P. australis observed in the west coast waters, was not confirmed for other affected areas. Lundholm et al. (1994) showed *P. seriata* produced DA at low temperatures, thus it could potentially represent a source of DA in colder, northern Scottish waters. The DA concentration of the 1999 Scottish isolates of *P. australis* (3–4 pg total DA $cell^{-1}$) compare closely with previous studies of *Pseudo-nitzschia*, as cellular DA levels are reported to range from 0.1–10 pg DA $cell^{-1}$ for most species studied to date (cited in Bates 1998). Previous studies of western North American *P. australis* have indicated comparatively high DA production capabilities (12–37 pg DA $cell^{-1}$, Garrison et al. 1992). However, our data are more consistent with estimates of 2.0 pg DA $cell^{-1}$ for New Zealand strains (Rhodes et al. 1996), and with unpublished data from Spanish and Irish *P. australis* strains (S. Bates, Fisheries and Oceans, Canada, pers. comm. 2000).

It is likely that the 1999 DA toxification, measured in December 1999, in Scottish king scallops occurred as a result of *Pseudo-nitzschia* blooms during the May to August 1990 period and not the result of continuous intake from toxic benthic sources (Bourne 1965), because *Pseudo-nitzschia spp.* concentrations were very low during October and December, and no significant quantities of living or dormant *Pseudo-nitzschia* cells were detected within the locations at time of sampling. Therefore, the current results support the hypothesis that high DA levels in scallops are a consequence of low rates of toxin catabolism as a result of low winter basal metabolic rates and reduced filtration activity, further influenced by colder waters and reduced food supply (Shumway & Cembella 1993).

The considerable degree of toxin variability observed among individual *P. maximus* and their body components was not unexpected and has been described for other shellfish species contaminated with DA and PSP toxins (White et al. 1993, Arevalo et al. 1998). Characterizing variation in toxin levels among individual species of the same area is necessary both for ecological considerations and for development of sound management protocols (White et al. 1993). The results do give an indication of the scale on which microhabitat differences influence ASP toxicity in *Pecten maximus* populations, because, despite wide individual variation, significant differences were found in all other tissue and gonadal toxin levels between groups of individuals only 25 m apart. Variation in bivalve toxicity is reported to result from an interaction of such factors as timing, persistence, and magnitude of toxic blooms, microgeographic variation in exposure to toxic cells because of bloom patchiness, the specific toxicity per cell, and toxin composition of the contaminating organism, environmental effects on scallop metabolism, and, perhaps, genotypic differences among scallop populations (Bricelj & Shumway 1998). However, the reasons for the few individual scallops retaining exceptionally large toxin burdens in all other tissue (>1,000–3,689 µg DA g^{-1}) are not known.

The ability to detect influences of scallop size parameters on DA accumulation may have been restricted by the limited size class (90–120 mm shell length; i.e., legal landing size) selected for use in the current study. Expanding the range of sizes used to

TABLE 4.

Mean and standard error (SE) of DA levels (μg DA g^{-1} wet weight) in the adductor muscle of *P. maximus* at each sample location and subsite.

Location and subsite	*n*	Mean	SE	Minimum	Maximum	CV (%)	95% CI	no. >20 μg g^{-1}
Loch Sligachan	30	1.254[b]	0.573	0.025	15.415	250	0.083–2.425	—
Tobermory Bay	40	0.156[a]	0.037	0.020	1.395	151	0.081–0.231	—
Area J5	100	0.581[b]	0.081	0.011	5.487	139	0.420–0.741	—
Loch Sligachan								
1	10	2.890[a]	1.630	0.030	15.42	179	−0.80–6.60	—
2	10	0.559[a]	0.219	0.045	2.310	124	0.06–1.05	—
3	10	0.313[a]	0.040	0.168	0.501	40.5	0.22–0.40	—
Tobermory								
1	10	0.192[a]	0.036	0.537	0.425	60.3	0.11–0.27	—
2	10	0.258[ab]	0.132	0.021	1.395	162	−0.04–0.56	—
3	10	0.046[b]	0.005	0.020	0.067	38	0.03–0.06	—
4	10	0.129[ab]	0.051	0.021	0.466	124	0.01–0.24	—
Area J5								
1	20	0.600[a]	0.111	0.086	1.962	82.6	0.37–0.83	—
2	20	0.238[b]	0.0345	0.063	0.592	64.7	0.16–0.31	—
3	20	0.526[bc]	0.273	0.085	5.487	231	−0.05–1.10	—
4	20	0.484[ab]	0.091	0.112	1.652	83.6	0.30–0.67	—
5	20	1.056[a]	0.235	0.225	3.962	99.7	0.60–1.50	—

Means from the same location with different superscripts are significantly different ($P > 0.05$, Kruskall–Wallis and Dunn's method). Ninety-five percent confidence intervals (CI), minimum and maximum levels of DA obtained, coefficient of variation (CV %) and number of individuals with adductor muscle toxin burdens >20 μg DA g^{-1} statutory limit are given.

include juveniles may indicate any allometric influences on DA toxin accumulation in *P. maximus*. Under controlled conditions, weight-specific DA toxicity has been demonstrated to be inversely proportionate to body size in mussels *Mytilus edulis* (Novaczek et al. 1992). However, faster detoxification rates per unit body mass in actively growing, smaller, or younger individuals, because of toxin dilution through growth, may mask any allometric relationships present (Bricelj & Shumway 1998).

A significant positive correlation was observed between toxicity of the gonad and that of all other visceral tissue. Although little is known about transfer of DA among tissues, its likely that gonadal toxicity is influenced by the level of digestive gland toxicity, via the intestinal loop, which passes through the gonad and may contain toxic feces. Cembella et al. (1993) demonstrated that PSP toxins are accumulated within gonadal follicles of *P. magellanicus*, even after the exclusion of the intestinal loop. However, the inherent wide individual variation precludes the ability to predict gonad toxicities reliably from routine ASP toxin monitoring of the viscera. Compared with other body components, the variation in adductor muscle toxicity was proportionately larger, and no correlation could be found between toxicity of the adductor muscle and that of all other tissue. The variance in toxicity values in adductor tissue may be attributed to one of, or a combination of, three sources: (1) natural variation in adductor muscle toxicity; (2) variable contamination of the tissue from digestive fluid, during dissection; and (3) analytical error close to the limits of detection. The mean CV accounted for by the detection method for all other tissue, gonad, and adductor muscle was ±11.8, 4.6, and 18.6%, respectively, indicating that the variability observed between individual scallops was not a result of analytical error. The extent to which toxic digestive fluid and exudates contaminates edible tissues should be established to ascertain the potential to reduce the ASP toxin burden by appropriate preparation of adductor muscle and gonad tissue and realize the necessity to standardize preparation of these tissues before testing.

During ASP events, the marketing of *P. maximus* digestive gland, mantles, and gills, poses a high risk to public health, which has an impact primarily on diver-based and cultivation industries supplying markets for whole scallops. However, to allow the marketing of the nontoxic edible component, scallop preparation techniques should be promoted, such as the immediate removal of toxic tissues and thorough washing of the edible component (having ascertained the gonad is safe to consume), and this practice should be regulated and conducted by skilled processing staff before the product reaches the consumer (Shumway & Cembella 1993; Curtis et al. 2000). Our results verify that strict regulatory and monitoring regimes should remain compulsory for the safe marketing of "roe-on" scallops. However, when gonad toxicities are greater than the regulatory limit, discarding of tissues that selectively sequester the DA toxin may provide an effective strategy to enable the marketing of adductor muscle, in conformity with the domestic "roe off" market of the United States and Canada (Bricelj & Shumway 1998).

The concentration of DA in gonad tissue varied by an order of magnitude (range 0.13–75.5 μg DA g^{-1}). Thus, if gonads with high toxicities were to be included in pooled samples, they could potentially elevate toxin levels significantly. This may explain why monitored toxicity at certain sites seemed to oscillate throughout the winter period (FSA pers. comm. 1999). Consequently, a large number of individuals should be included in composite samples to reflect mean population toxicity accurately. However, in species where toxicities are extremely variable, it is the consensus that monitoring tissues on an individual basis proves more informative in developing mitigating strategies for harmful algal bloom management. Curtis et al. (2000) were able to propose site-specific recommendations for management, on the basis of large differ-

TABLE 5.

Concentration of DA in three *Pseudo-nitzschia* cultures established from the 1999 ASP event (pg DA cell^{-1}) of intracellular and extracellular fractions and combined total). Cultures harvested at three weeks.

Species	Domoic acid content (pg cell^{-1})		
	Intracellular	Extracellular (supernatant)	Total (combined)
P. australis (isolate 1)	1.32	2.95	4.27
P. australis (isolate 2)	1.20	2.19	3.39
P. pungens	nd	nd	0.00

nd = not detected.

ences in PSP toxicity among geoduck clams, *Panope abrupta*, of different depths and harvest tracts. Data describing individual variability of gonad toxicity within localities allow subpopulations with a low frequency of individuals of elevated gonadal toxicity to be distinguished (as seen in the current study); therefore, they permit evaluation of the level of risk, gonad tissue from specific locations with respect to its rate of consumption, poses to human health. The use of risk assessment models should be considered to assess scallop toxicity with respect to rate of consumption by humans, to continue to maintain public safety standards while at the same time ensuring optimum utilization of the high-quality king scallop resource.

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STRANDING OF SCALLOPS RELATED TO EPIPHYTIC SEAWEEDS ON THE COAST OF NORTHERN CHILE

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ABSTRACT More than two million scallops (*Argopecten purpuratus* Lamarck) were stranded on the beach at Tongoy Bay, northern Chile (30°20′S) in March 1999 during a period of unusually strong wave action. The scallops, which were stranded in living condition, had frondose algae attached, principally *Ulva lactuca* Linneaus, to the upper valve. Settlement and growth of the algae on the scallop shells facilitated the stranding due to the large surface area thus exposed to water movement. A preponderance of larger size classes of scallops fouled with algae were stranded, suggesting that the epibionts decreased the chance of "escape" from the effects of water movement in relation to the size of the individuals.The stranding represented about 20% of the population of the bed. It is suggested that nearshore areas were mainly affected, where light conditions promoted the rapid growth of the epiphytic seaweeds.

KEY WORDS: scallop stranding, algal epiphytism, mass mortality, Chile

INTRODUCTION

Stranding and mass mortalities on the shore are frequent occurrences and affect distinct groups of marine organisms. There is a great deal of information on the stranding of marine mammals and turtles (Raga et al. 1991, Blochet al. 1997, Berrow & Rogan 1997). However, in the case of marine invertebrates, the literature is limited, particularly as it relates to Chile. For invertebrate strandings, many different causes are suggested. Natural mortality is cited for the strandings of the cnidarian *Velella velella* in Oregon (Kemp 1986). Some strandings are selective and affect a fraction of the population, as reported for the horseshoe crab (*Limulus polyphemus*), in which stranding is associated with senescence and parasitism in some individuals (Penn & Brockman 1995). In other cases it is related to changes in the environment, such as the mortalities produced by the El Niño phenomenon. This phenomenon has been implicated in strandings of the pelagic crab (*Pleuroncodes planipes*) (Aurioles et al. 1994), as well as mollusks (*Mesodesma donacium*) and brown algae of the genus Macrocystis (Arntz & Valdivia 1985, Arntz et al. 1985). Major storms and high tides may cause stranding, as in the case of the lobster (*Homarus americanus*) on the coast of Prince Edward Island, Canada (Maynard & Chiasson 1988) and the yellow clam (*M. mactroides*), of which millions were stranded in Argentina (Oliver et al. 1971). Other strandings include those related to pollution, such as oil spills (Dyrynda et al. 1997).

Strandings of scallops, resulting from strong winds and storms or due to consequences of disease, parasitism, or senescent states, have been reported (Tettelbach 1985, Orensanz et al. 1991). Tettelbach (1991) reported strandings of the scallop *Argopecten irradians* (Lamarck, 1819) in the Poquonock River in Connecticut, which was associated with strong wave and tidal currents. If the scallops are stranded live, they die due to desiccation, bird predation, and human harvesting.

Tettelbach (1991) included the anecdotal case of a stranded scallop having an abundant covering of the alga (Codium) on its shell. However, this is not an isolated phenomenon, as shown by the report of Kelley and Kirby (1981), who related an extensive stranding of the bay scallop on Nantucket Island, Massachusetts due to *Codium fragile*. Galtsoff (1964) called the epiphytic Codium "oyster thieves." Ansell et al. (1988) related a major stranding of the sandy bottom clam *Donax vittatus* (da Costa) in Dornach, Scotland, to the growth of algae on their shells. Similarly, Orensanz et al. (1991) mentioned that the risk of stranding in *Chlamys tehuelcha* (Patagonia, Argentina) was increased by the presence of epibionts.

The present report describes a stranding of the scallop *A. purpuratus* (Lamarck) on the coast of northern Chile, produced by swell-induced waves, and the facilitation of the stranding by the presence of epibiotic algae on the scallops.

MATERIALS AND METHODS

Study Area

Observations were made at Puerto Aldea (30°17′S, 71°36′W), a small locality at the extreme southern end of Tongoy Bay, which is protected from the predominant southwest winds by Lengua de Vaca Point (Fig. 1). However, with north winds it experiences a major swell. Off Puerto Aldea, the bottom is composed of sand and rocky areas and a sector covered by the seagrass (*Heterozostera tasmanica*), which forms a bed of approximately 1.5 hectares (Phillips et al. 1983, Gonzalez 1992). The scallop (*A. purpuratus*) is distributed over the entire Puerto Aldea area (Stotz & Gonzalez 1997), which is protected and managed by local fishermen.

Methods

During a period of unusually high swells on the northern coast of Chile in March 1999, 221 stranded scallops (*A. purpuratus*) were collected from a strip of beach at Puerto Aldea. The shell height (dorsoventral length) of the scallops was measured to ±1 mm. The total fresh weight of the scallops was obtained using the relationship of size to weight determined for this region by Stotz and Gonzalez (1997): Weight (g) = $2.897 \times 10^{-4} \times$ Height $(\text{mm})^{2.905}$.

The occurrence of algae attached to the shells of each individual was recorded, both in cases where the entire thallus was present or where there was evidence of algae (e.g., holdfast). Given the characteristics of the algae encountered, our work cen-

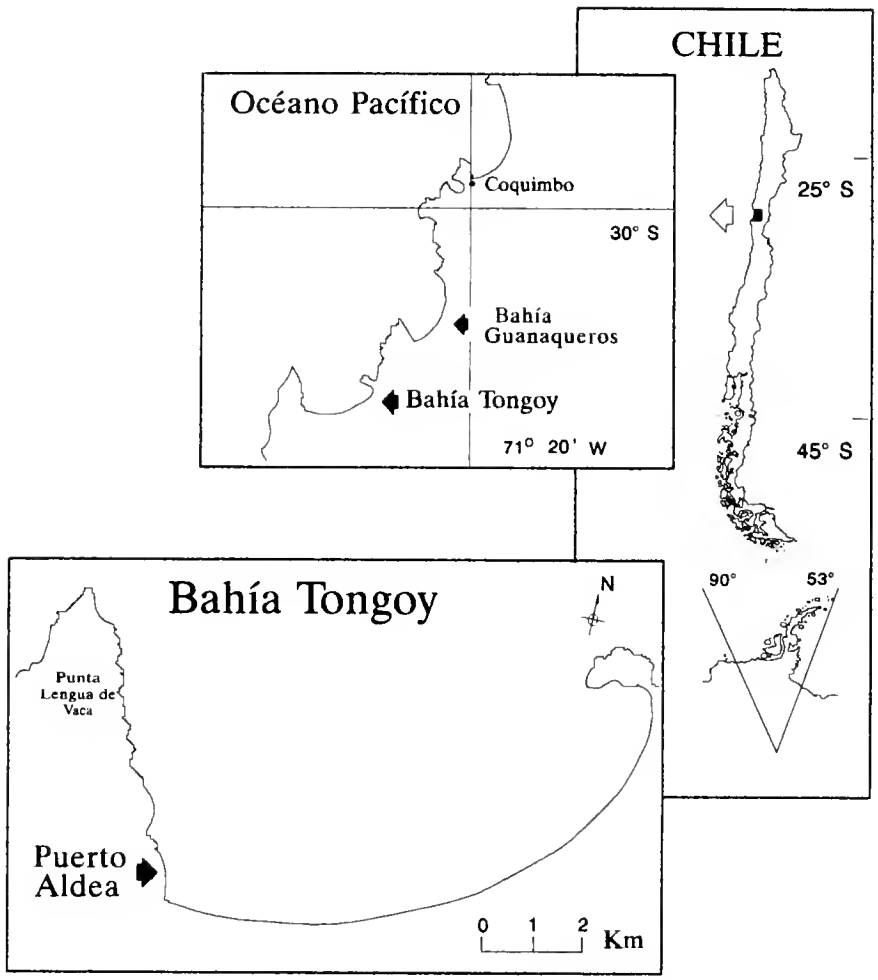

Figure 1. The location of the scallop stranding at Tongoy Bay, northern Chile.

tered on the most abundant alga, *Ulva lactuca* Linneaus (1753). Algae were measured for maximum length from adhesive disc to the end of the longest frond (±1 mm), and wet weights were measured to ±0.1 g on a portable balance. Algal surface area was determined using the equation 1 g wet weight of *U. lactuca* = 89 cm^2, obtained by weighing 30 algal pieces cut to 16 cm^2 (0.18 g).

An estimate of the magnitude of the stranding was obtained by personal interviews with fishermen at Puerto Aldea. Their determination was made on the basis of the number of scallops that they collected and from their estimates of the numbers of scallops removed from the beach by people from outside the Puerto Aldea community.

RESULTS

Stranded Scallops

Sixty-five percent of the stranded scallops bore an algal thallus on the shell, with a further 20% showing the presence of a holdfast or a partial algal thallus. The remainder of the scallops had no macroalgae attached to their shells.

Although a wide size range of scallops were stranded (Fig. 2), most of the scallops collected ranged from 40–80 mm in shell height. Scallops with algal epibionts were somewhat larger than those without epibionts (Table 1). A median *t* test showed significant differences in the sizes of the scallops in these groups (t value$_{(73)}$ = 5.1338, $P < 0.001$).

Characteristics of Algal Epibionts

Algae on the scallops included principally *U. lactuca*, with the occurrence of only seven individuals of *Cryptonemia obovata* (J. Agardh, 1876) and one of the brown alga *Desmarestia ligulata* (Lightfoot) (Lamouroux, 1813).

Fronds of Ulva on the scallop shells had a broad range of sizes,

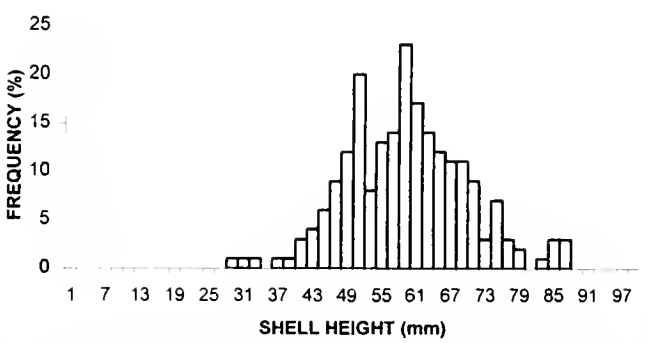

Figure 2. Size–frequency distribution of *Argopecten purpuratus* stranded at Puerto Aldea, Chile in March 1999.

TABLE 1.

Percentage of different size groups of scallops (*Argopecten purpuratus*) stranded at Puerto Aldea, Tongoy Bay, Chile, in March 1999.

	Range of sizes (shell height, mm)											
	Total		0–20		21–40		41–60		61–80		81–100	
Epibiont status	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
With algae	190	80.2	0	—	3	60	89	79.5	81	91	7	100
Without algae	33	19.8	0	—	2	40	23	20.5	8	9	0	—

ranging from 34–971 mm in length, with an average length of 378 mm (Fig. 3). Fronds frequently exceeded 170 mm in length. The surface area of the fronds ranged from 98–6,803 cm^2, and the weight ranged from 1.1–76.1 g.

Scallop–Algae Relationship

A tendency was observed for the algae on the scallop shells to be larger on the larger sizes of scallop. Although there was a broad dispersion of values, this relationship was statistically significant via linear regression ($F_{0.05(1,136)} = 46.5$, $P < 0.001$) (Fig. 4).

Magnitude of the Stranding

Because of their commercial value, the scallops did not accumulate on the beach; they were harvested by a great number of people and taken away for sale. A survey of 43 persons at Puerto Aldea who harvested scallops produced a total of 217,550 scallops. This harvesting extended over the 4 days during which the stranding occurred. Estimates by the fishermen's association of Puerto Aldea indicated that about 1,700 other people visited the area of the stranding and removed a total of about 2 million scallops. On the basis of the sizes of scallops stranded, we estimated the total fresh weight of scallops removed to be about 100 metric tons.

DISCUSSION

The higher incidence of stranded scallops bearing algal epibionts than those without epibionts demonstrated the importance of algae to the stranding of scallops during the period of heavy swell. Observations made by diving after the passage of the stranding event (M. Aguilar, pers. observ.) showed an almost complete absence of algae-bearing scallops remaining in the bed, suggesting that essentially all epibiont-affected scallops had been beached by the waves. It was probable, due to the light requirements of the green algae, that the beached scallops came mainly from shallow areas near the shore, which would be more subject to water movement. However, it is also possible that the swell ripped off the epiphytic Ulva fronds from the shells of the scallops.

Although the stranding was associated with heavy swells that affected Puerto Aldea when winds of about 10 m/s and waves of about 2 m in height occurred for several hours (Chilean Navy data), the stranding was facilitated by the presence of algae on the scallop shells. For example, an alga 50 cm in length with a surface area of 1,700 cm^2 on a scallop 6 cm in height represents an increment of 40× the surface area of this scallop normally exposed to water movement.

The fact that large scallops not bearing epibionts were generally absent from the stranding suggests that these scallops were able to escape the effects of water movement by virtue of their size (weight), whereas scallops with algal growth on the shells were transported to the shore. This concurs with the observations of Witman and Suchanek (1984) that the presence of algae such as *Lauminaria saccharina* and other epibiota on the shells of *Mytilus edulis* and *M. californianus* significantly increased their risk of being dislodged by currents.

The stranding may be related to climatic alterations related to the El Niño phenomenon, both in reference to the heavy swells and the abundance of green algae occurring in this period. Also, changes in sea state that produce unusual wave action may be accompanied by rises in sea surface temperature associated with El Niño currents and provide improved conditions for algal growth. For example, Arntz et al. (1985) reported blooms of frondose green algae, which grew rapidly during the El Niño event of 1982–83 and dominated rocky shallows on the coast of Peru.

An evaluation of the scallop bed at Puerto Aldea carried out 2 mo after the stranding (Stotz & Gonzalez, unpub. technical report) showed the presence of about 8 million scallops. The stranding

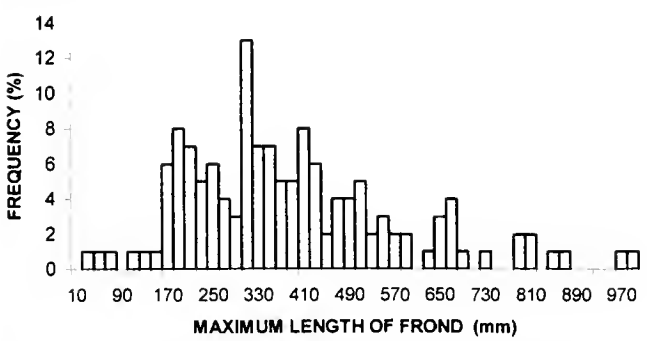

Figure 3. Size–frequency distribution of algal thalli (*Ulva lactuca*) attached to *Argopecten purpuratus* stranded at Puerto Aldea, Chile.

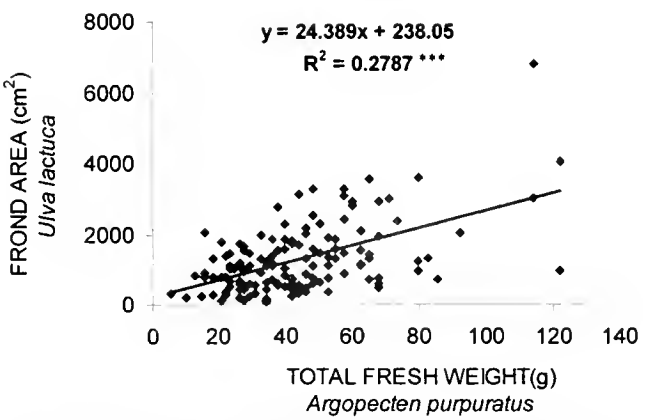

Figure 4. Relationship between size of stranded *Argopecten purpuratus* and the surface area of algae (*Ulva lactuca*) attached to their shells. ***Linear regression was significant at $P < 0.001$.

therefore represented a loss about 20% of the population (previous to the swell period) from this bed. However, in addition to the observed effects of the stranding of scallops on the natural population, this event could have a negative effect on the perception of the local fishermen about the benefits of protecting the resource. Nature took away almost 20% of their possible income. Although the stranded scallops were harvested, it was mainly by foreign people, not by members of the Puerto Aldea community. This needs to be evaluated.

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REPRODUCTION, POPULATION STRUCTURE, AND RECRUITMENT LIMITATION IN A BAY SCALLOP (*ARGOPECTEN IRRADIANS* LAMARCK) POPULATION FROM NEW JERSEY, USA

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ABSTRACT A bay scallop, *Argopecten irradians irradians* (Lamarck 1819), population was studied in Little Egg Harbor, New Jersey, USA to investigate the distribution, reproduction and genetic structure of the population because virtually nothing is known about this species in this region. *Argopecten irradians irradians* densities varied among eelgrass (*Zostera marina* Lamarck) beds in 1998 (range 0.12–1.0 individuals per 10m transect), but were virtually absent from the region in 1999. The absence of adults in 1999 may have been due to limited spat recruitment and survival in the fall of 1998, which may be attributed to a reduction in above ground biomass of *Z. marina* habitat. Genetic analysis (using mtDNA) indicated that this New Jersey population was intermediate between New York and North Carolina populations. Based on these data and the historical landings of bay scallops in New Jersey, it is probable that a small self-seeding population exists. Assessment of reproductive cycles during 1998 showed two potential peaks in reproductive condition: one occurred during June when a maximum Gonadal-Somatic Index was recorded (24.7%) and the second was during October when a visual gonadal condition index indicated a majority of scallops in post-spawn condition. Few adult bay scallops were encountered in 1999 ($n = 8$) to assess reproductive cycles, but two large individuals collected during July showed a post-spawn gonadal condition. However, during both 1998 and 1999 settling juveniles (< 15 mm shell height) were only recorded in October, suggesting that recruitment to the population during these years resulted from the late summer-early fall spawn. Given the variability observed over the two years, future studies should concentrate on factors influencing inter-annual variation in abundance of this New Jersey population.

KEY WORDS: bay scallop, *Argopecten irradians*, reproduction, genetic structure, recruitment

INTRODUCTION

The bay scallop (*Argopecten irradians*) was once a common and often abundant member of shallow marine communities along the Atlantic and Gulf coasts of the United States and a prized commercial and recreational shellfish. Clarke (1965) identified three distinct sub-species of *A. irradians* based on morphological characteristics, but recent morphological and genetic studies have suggested a fourth (Blake and Graves 1995, Marelli et al. 1997, Wilbur and Gaffney 1997). The northern sub-species, *A. i. irradians* ranges from Massachusetts to New Jersey where it is thought to intergrade with the southern subspecies *A. i. concentricus*. *Argopecten irradians concentricus* was originally described to range along the mid-Atlantic coast and into the Gulf of Mexico (Clarke 1965, Waller 1969). Recent studies, however, have shown that bay scallops from the Florida gulf coast and from North Carolina are as different from one another as are the northern and southern subspecies on the Atlantic coast, indicating that these populations should be considered distinct subspecies (Blake and Graves 1995, Marelli et al. 1997, Wilbur and Gaffney 1997). The fourth subspecies, *A. i. amplicostatus*, is occasionally reported from the gulf coast of Texas and extends to an undefined southern limit south of the Yucatan Peninsula. This sub-species has been described solely on the basis of morphological analysis and the scarcity of animals in recent years has constrained the incorporation of representatives in the aforementioned genetic surveys. A preliminary analysis of the mitochondrial DNA variation does not support the separation of *A. i. amplicostatus* from other Gulf populations despite the clear demonstration of genetically determined differences in morphology (Wilbur 1995, Wilbur and Gaffney, 1997). As such, the taxonomic status of the western gulf form of *A. irradians* is unclear.

Bay scallops are intimately tied to seagrass beds, which they use as a primary settlement site (Gutsell 1930, Eckman 1987). Specifically, scallops settle and cling to blades via byssal threads until they are too large to remain suspended (Thayer and Stuart 1974). During this life stage, the seagrass canopy provides protection from benthic predators (Pohle et al. 1991). However, reduced growth rates of juvenile scallops climbing higher on blades (Ambrose and Irlandi 1992), suggests that this behavior may represent a trade-off between growth and mortality. Ultimately, recruitment to the adult population may be determined by predation at this juvenile stage (Strieb et al. 1995). Because bay scallops recruit to seagrasses and use them as attachment sites (Thayer and Stuart 1974), the loss of habitat during the eelgrass (*Zostera marina*) wasting disease in the 1930s (see den Hartog 1987) is thought to have severely limited populations in many regions, while eliminating them from others. Although eelgrass has made recoveries in the subsequent decades (den Hartog 1987), bay scallops have not returned in significant numbers to many areas where they once were abundant. In coastal New Jersey, eelgrass has returned and is relatively abundant in shallow water, yet in the last several decades bay scallop densities remain below a fishable population size (Ford 1997).

Historically, bay scallops were abundant and commercially fished in New Jersey, USA. The first available landing records were collected in 1956, when 52,300 bushels were harvested with an estimated value of $287,000. Continued success of scallop populations for the next 12 years yielded 317,000 bushels valued at over $1 million (Ford 1997). Subsequently, commercial bay scallop harvests were only recorded for 1973 and 1974. Despite its

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local importance, little information exists on the ecology and population structure of New Jersey bay scallops, particularly since the collapse of the commercial fishery. In recent years it was generally felt that bay scallops no longer occurred in New Jersey waters. The observation of numerous scallops in 1998 in Little Egg Harbor, New Jersey prompted questions regarding the density and reproductive periodicity of this population, as well as its origin. New Jersey is thought to be the point of contact between the *A. i. irradians* and *A. i. concentricus* sub-species, and the return of scallops suggests recruitment of larvae from extant populations north or south of New Jersey, or from a small, remnant local population. It was the goal of this research to investigate the distribution, reproduction and genetic structure of New Jersey bay scallops.

STUDY SITE

Investigations were conducted during 1998 and 1999 in Little Egg Harbor, New Jersey, U.S.A. (39°35′N, 74°14′W; Fig. 1), which is located in the central portion of the Mid-Atlantic Bight. Little Egg Harbor is a relatively unimpacted region of coastal New Jersey and is part of the Jacques Cousteau National Estuarine Research Reserve (Psuty et al. 1993). It is a polyhaline estuary protected by a barrier island. It is relatively shallow (average depth at MLW = 1.7 m, Durand 1984) and submerged aquatic vegetation covers approximately 1,305 hectares of the bottom (Bologna et al. 2000). Seasonal water temperatures range from −2°C to 28°C (Able et al. 1992) with an average tidal range of about 0.7 m (Chizmadia et al. 1984).

MATERIALS AND METHODS

Population Assessment

Argopecten irradians densities were assessed at four sites in Little Egg Harbor, New Jersey during May 1998 and 1999. Density was determined using 10-m × 1-m transects haphazardly lain out in shallow (< 1.5m depth) *Zostera marina* beds (Table 1,

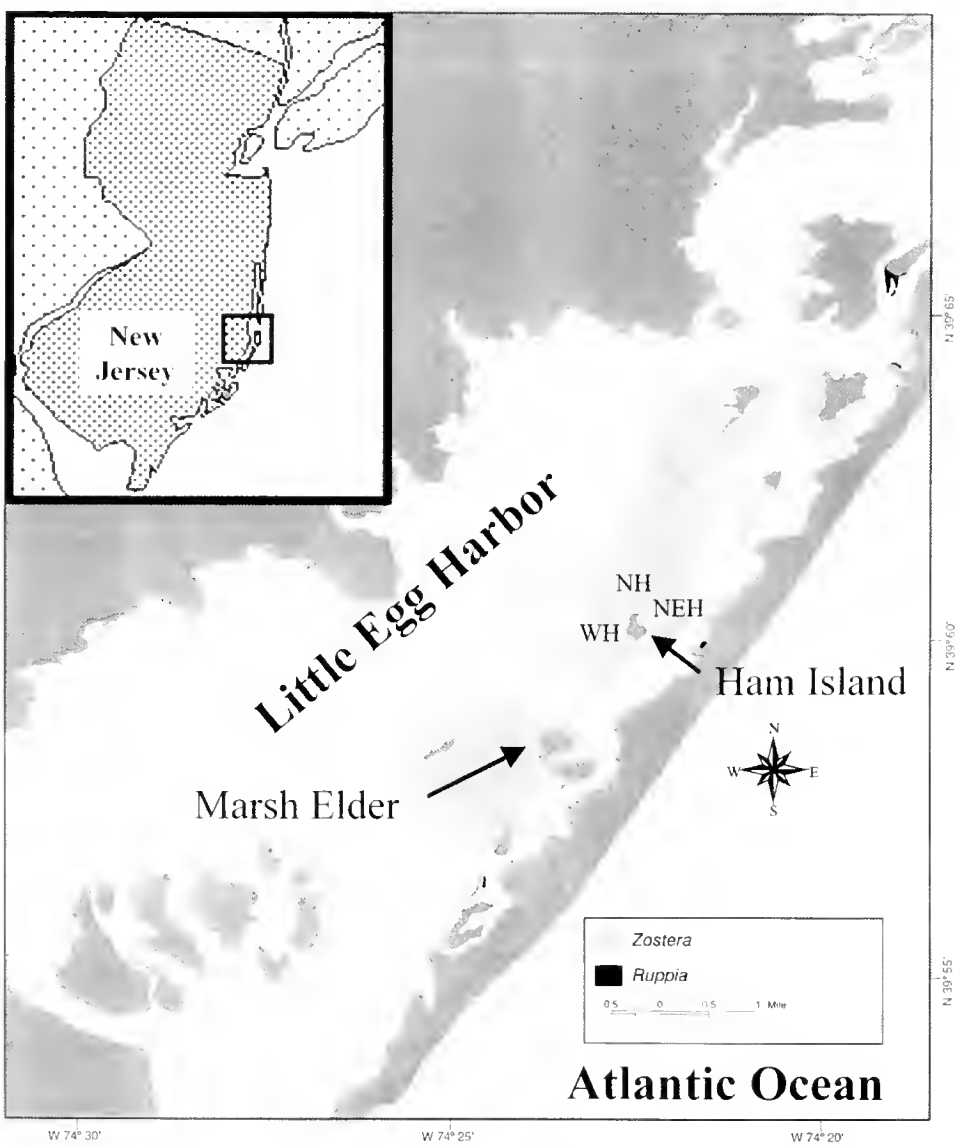

Figure 1. Little Egg Harbor, New Jersey (39 35′N, 74 14′W) with locations of Marsh Elder and Ham Island and dominant vegetation types. Abbreviations associated with Ham Island refer to sites used for population density estimates: WH = West Ham Island, NH = North Ham Island, NEH = Northeast Ham Island.

Fig. 1): (1) West Ham Island (n = 16 transects, 1998; n = 10, 1999), (2) North Ham Island (n = 17; n = 10), (3) Northeast Ham Island (n = 14, n = 10), and (4) Marsh Elder Island (n = 8, n = 10). They were sampled by snorkeling the length of the transect and collecting all scallops within 50 cm of the transect line (1-m width). Scallop abundance was compared among sites and between years using a two-way ANOVA with site and year as independent variables and scallop abundance as the dependent variable. The size structure of the population was assessed monthly from visually located field-collected individuals during 1998. Size frequency distributions were generated for months in which at least 16 individuals were collected to assess the potential age structure of the population.

Reproduction

Visual inspection of the gonad condition and calculation of a Gonadal-Somatic Index (GSI) assessed scallop reproduction from collections on 10 dates from April 1998 to August 1999. Scallops were frozen and returned to the laboratory where shell height was measured to 0.05 mm and gonadal and somatic tissues were then dissected out. Visual condition of gonad material for scallops was determined for each individual and assessed as undeveloped, ripening, very ripe, or post-spawn following the protocol of Bologna (1998). Reproductive and somatic tissues were then dried at 60°C for 72 hours and weighed (g dry weight). The GSI was calculated for each scallop using the following equation: GSI = (gonad dry weight/total dry weight) *100. Evidence of reproductive success was assessed by collection of small juveniles (<15 mm shell height).

Recruitment Habitat Assessment

Characterization of bay scallop habitat was assessed by collections of benthic cores in *Zostera marina* beds during March, April, May, June, July, and October 1998 to determine shoot density and plant biomass (n = 6 cores/month). The coring device (15.24 cm diameter (0.01824 m^2)) was pushed into the substrate to a depth of 25 cm, capped, and removed from the sediment. Samples were frozen and returned to the laboratory. Shoot abundance was determined and samples were separated into *Z. marina* above ground (shoots) and below ground (rhizomes and roots) portions, and an algal-detrital fraction. Above ground *Z. marina* and algae-detritus were dried to constant weight at 80°C, then ashed at 500°C for eight hours to determine ash free dry weight (AFDW). Shoot abun-

TABLE 1.

***Argopecten irradians* density comparisons among sites in Little Egg Harbor, New Jersey (see Fig. 1) for 1998 and 1999.**

Site	Density n	Density 1998	Density n	Density 1999
West Ham Island (WH)	16	0.12 ± 0.34	10	0 ± 0
North Ham Island (NH)	17	1.00 ± 0.79*	10	0 ± 0
Northeast Ham Island (NEH)	14	0.21 ± 0.42	10	0 ± 0
Marsh Elder Islands	8	0.25 ± 0.46	10	0 ± 0

N indicates the number of transects conducted at each site during each year. Density values represent mean number of scallops encountered per 10-m transect ± one standard error.

* Represents significantly greater scallop density for North Ham Island site compared to others ($P < 0.0001$).

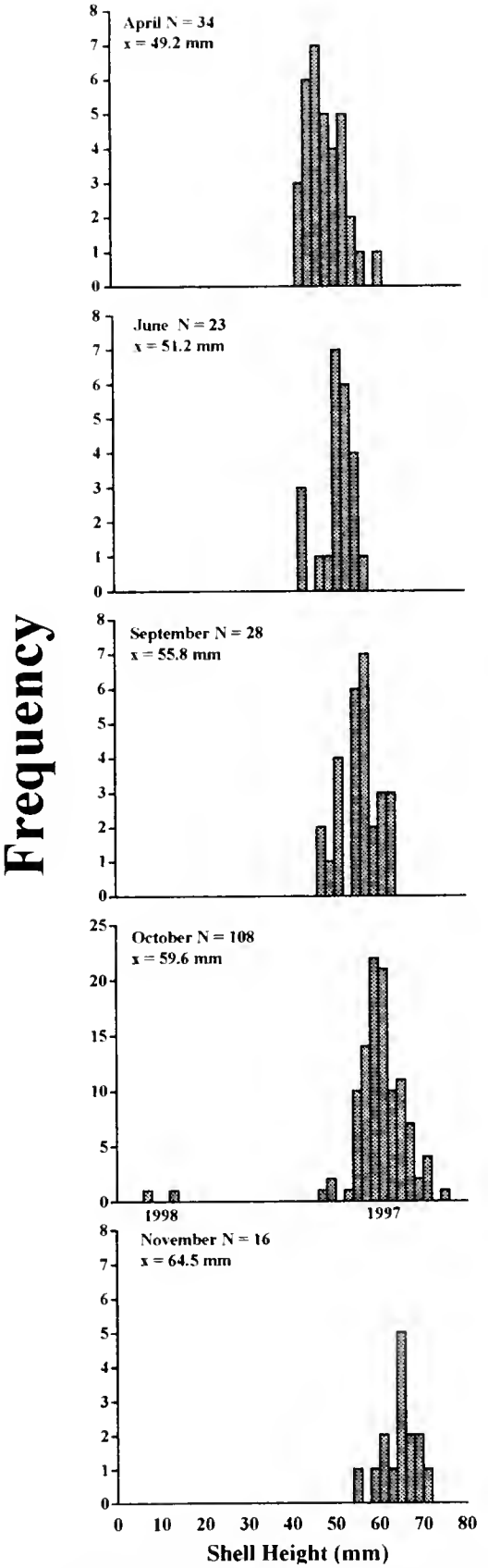

Figure 2. Bay Scallop Size Frequency Distribution. Size distributions for 1998 collections based on scallop shell height measurements (mm). Sample size (N) and average shell height (x) given for each month. Note y-axis scale change for October and the identified year classes for scallops represented in the sample.

TABLE 2.

Scallop reproductive potential: Visual gonad condition index.

	n	Mean shell height (mm)	Undeveloped	Ripe	Very ripe	Post-spawn
1998						
April	2	43.0	2	0	0	0
June	13	52.3	0	3	10	0
July	10	50.0	1	6	3	0
August	4	55.6	0	2	2	0
September	15	55.7	1	13	1	0
October	20	58.6	0	0	2	18
November	16	64.3	0	0	0	16
1999						
May	3	70.0	0	2	1	0
July	4	55.1	0	0	2	2
August	1	43.3	0	0	1	0

N indicates the number of *Argopecten irradians* collected on each date for reproductive assessment. Values in the table represent the number of individuals exhibiting each condition for a sample.

dance data were square-root transformed and plant biomass data (*Z. marina* and algae-detritus) were log transformed before analysis. Shoot abundance, above ground biomass, and algae-detritus biomass were compared among months using a General Linear Model and significance testing was performed using an LSMEANS procedure with alpha = 0.05 (Littell et al. 1991).

Genetic Stock Assessment

Total DNA was extracted from 20 scallops collected in Little Egg Harbor during 1998 (PureGene extraction Kit, Gentra Systems, Inc) and amplified using primers specific for an 833bp fragment of the 12s ribosomal and NADH dehydrogenase 1 subunit regions of the mtDNA genome. Polymerase chain reaction (PCR) amplifications were carried out in 50μl reaction volumes and were subjected to an initial denaturation step of 3 min at 94°C, followed by 40 cycles consisting of 30 sec at 94°C, 30 sec at 53°C and 1 min at 72°C. Each product was digested with 9 restriction endonucleases (*Alu* I, *Ban* II, *Bgl* I, *Bsi*HKA I, *Rsa* I, *Hinf* I, *Scr*F I, α*Taq* I and *Tsp*509 I) following manufacturer's protocols (New England Biolabs, Beverly, MA). Digestion products were electrophoresed on 2% agarose gels and visualized using ethidium bromide.

Restriction fragment patterns were analyzed using the REAP (McElroy et al. 1992) and compared with data previously collected on scallop samples from New York (East Hampton), North Carolina (Core Sound, Bogue Sound), and Florida (Florida Bay, Homosassa, St. Joseph's Bay). For each of the seven populations sampled, nucleotide divergences were estimated and corrected for within population variation (Nei 1987). Sample haplotype frequency distributions were tested for heterogeneity using contingency tables and a randomized chi-square test of independence (Roff and Bentzen 1989). Population structure among scallop samples was analyzed using a hierarchical analysis of molecular variance (AMOVA, Excoffier et al. 1992).

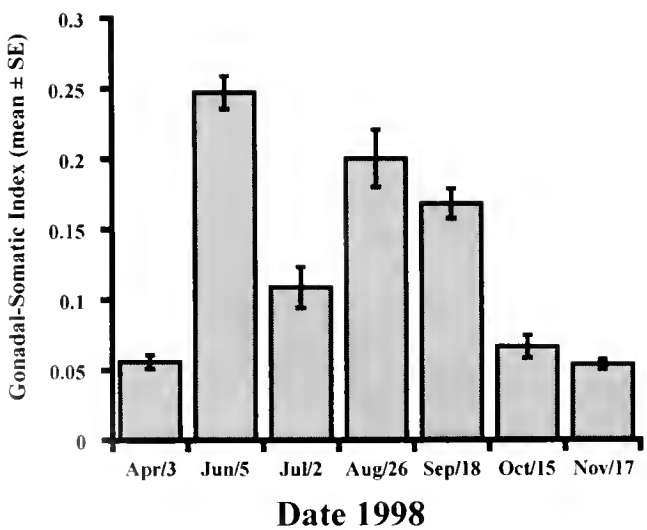

Figure 3. Seasonal Reproductive Assessment for 1998. Values represent calculated mean Gonadal Somatic Index ± Standard Error for scallops collected. See Table 2 for visual gonad condition index.

RESULTS

Population Assessment

During 1998, bay scallop densities were significantly greater from all Little Egg Harbor study sites compared to 1999 ($F_{1,87} = 24.6$; $P < 0.0001$), because no scallops were collected during 1999 transects (Table 1). As such, a significant interaction occurred between year and site in the two-way ANOVA ($F_{3,87} = 5.6$; $P < 0.001$). Therefore, we conducted a post-hoc ANOVA comparing scallop abundance among sites for 1998. Results indicated significant among site differences in scallop density ($F_{3,51} = 8.6$; $P < 0.0001$), with density significantly greater for the North Ham Island site (1.0 individual 10 m^{-2}) compared to the other sites (Table 1). Sufficient population samples were collected during April, June, September, October, and November 1998 to assess the size structure of the population (Fig. 2). Based on these data, a single year class (45–75 mm shell height) was identified but a few individuals of a second year class (6–12 mm shell height) occurred in October.

Reproduction

Visual assessment of gonad condition in 1998 suggested that spawning occurred during September and early October, as evi-

denced by the prevalence of post-spawn individuals in October (Table 2). However, this result differs with the assessment of GSI, which showed maximum values in June (Fig. 3), corresponding with 75% of the individuals showing a very ripe gonad condition (Table 2). Despite these very high GSI values, the presence of settling individuals (<15 mm shell height $n = 3$) was noted only for October. This was true for both 1998 (6 and 12 mm shell height, Fig. 2) and in 1999 (9 mm). Based on these preliminary data, the peak recruitment period for New Jersey *Argopecten irradians* may be early fall.

Recruitment Habitat Assessment

During 1998 significant losses of recruitment habitat, *Zostera marina*, occurred in Little Egg Harbor. Macroalgae (e.g., *Ulva*, *Gracilaria*, *Codium*) increased in abundance in eelgrass beds, blanketing the bottom. This led to significant increases in algal biomass by June, with significant loading of algal and detrital material by July and continuing through to October ($F_{5,25} = 13.3$, $P < 0.0001$, Fig. 4). These changes to the system resulted in significant declines in eelgrass shoot density and biomass by July 1998 ($F_{5,25} = 11.4$; $P < 0.0001$, $F_{5,25} = 8.9$, $P < 0.0001$), with complete elimination of above ground *Z. marina* biomass in the study area by October 1998 (Fig. 4).

Genetic Stock Analysis

A total of 22 haplotypes were resolved in the analysis of 120 *Argopecten irradians* from the six populations between New York and Florida (Table 3). Haplotype diversity (the probability that a pair of individuals sampled from a population were different in mtDNA haplotype) was moderate, averaging 0.53 (range from 0.1 in Rabbit Key, Florida to 0.82 in New York). Between-population nucleotide divergence estimates averaged 0.46% but did not show a clear correlation with geography. Curiously, the most northern populations (NY and NJ) exhibited lower divergence relative to Florida populations (Florida Bay, Homosassa and St. Joseph's Bay) than when compared to the geographically closer North Carolina (Core and Bogue Sound) populations (Table 3). The New Jersey sample was equally distant from its adjacent population: 0.066% relative to North Carolina, 0.068% relative to New York. Haplotype frequency distributions were significantly different among surveyed populations ($\chi^2 = 250.53$, $P < 0.0001$). Pair-wise comparisons revealed a lack of significant difference between the New Jersey and North Carolina samples ($\chi^2 = 29.78$, $P = 0.168$) but highly significant differences between New Jersey and New York ($\chi^2 = 23.01$, $P < 0.0001$).

Three working hypothesis regarding population structure in bay scallop were tested using AMOVA. The greatest amount of variation was accounted for with the New Jersey sample grouped with the North Carolina samples (alternate hypotheses grouped New Jersey with New York, or as a distinct New Jersey entity), supporting the result of the analysis of the haplotype frequency distributions. The majority of the variation (59.4%) was attributed to differences among regions (regions defined as North Atlantic (NY), South Atlantic (NJ, NC) and Gulf (FL)) while only a small fraction (1.8%) of the variation was accounted for by genetic differences among samples within defined regions (Table 4).

DISCUSSION

For species of economic value, assessing population structure and reproduction is essential for both wise management of healthy populations and conservation and enhancement of endangered populations. For bay scallops in New Jersey, some information exists regarding the commercial nature of the fishery and landings from preceding decades (Ford 1997), but virtually nothing is known about the ecology of this species. One feature of bay scallop ecology that is relatively well known for many other populations is reproductive effort. Our results indicate that bay scallops in New Jersey spawn in late summer or early fall (Table 2, Fig. 2), and recruit to eelgrass habitat in October, as evidenced by collection of a few newly recruited individuals in both 1998 and 1999. However, based on the assessment of gonadal condition index (Table 2) and the maximum GSI values (Fig. 3), we believe that scallops may also show a minor late-spring spawn. Although no recruits were collected during the summer, the visual gonad condition index suggested post-spawn conditioning of bay scallops from July 1999 (Table 2). This pattern of reproduction appears to be opposite of populations from New York, which show maximum reproductive effort during May through August, with only minor contributions during the fall (Tettelbach et al. 1999). However, it is similar to the reproductive pattern of bay scallops from North Carolina, which exhibits a strong fall spawn and a much less well documented early spring event (Peterson et al. 1989).

Given that bay scallops may suffer high mortality after spawning (Marshall 1963), significant research has focused on reproduction, reproductive conditioning and changes in biomass associated with gonad development (Bricelj et al. 1987a, Bricelj et al. 1987b). It has been shown that scallops show distinct peaks in spawning activity and these peaks differ temporally based on latitude of the populations (Sastry 1970, Barber & Blake 1983, Crenshaw et al. 1991). Although scallops do show peaks in reproductive activity, recruiting individuals (< 15 mm shell height) have been reported throughout the year, suggesting that trickle spawning may occur in some locations (Gutsell 1930, Bricelj et al. 1987b, Bologna 1998). Although our data are limited in scope, we surmise that only the

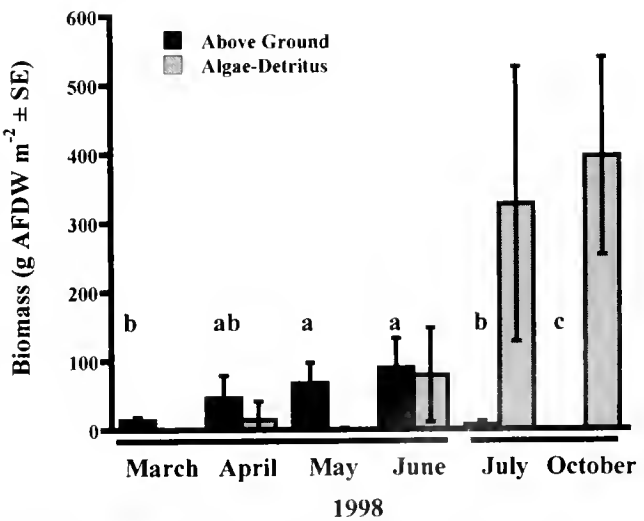

Figure 4. Seasonal distribution of *Zostera marina* above ground biomass and algal-detritat biomass from Little Egg Harbor, NJ during 1998. Values represent mean biomass expressed as gram ash free dry weight (g AFDW) per square meter ± Standard Error. Differing letters above bars represent significant differences in above ground *Z. marina* biomass among means for dates of collection ($P < 0.05$). Separated lines represent significant differences in Algae-Detritus biomass among months of collection.

TABLE 3.

Percent nucleotide divergence (Nei 1987) among populations of *Argopecten irradians* based on RFLP analysis of an 833bp mtDNA fragment (9 restriction enzymes).

	East Hampton	Little Egg Harbor	Bogue Sound	Core Sound	Rabbit Key	Homosassa	St Joseph Bay
East Hampton, NY	0.0000						
Little Egg Harbor, NJ	0.1307	0.0000					
Bogue Sound, NC	0.2813	0.0911	0.0000				
Core Sound, NC	0.3370	0.0625	−0.0335	0.0000			
Rabbit Key, FL	0.9339	1.0330	1.2570	1.3423	0.0000		
Homosassa, FL	0.4836	0.7250	0.9143	0.9738	0.0657	0.0000	
St Joseph Bay, FL	0.5290	0.6081	0.8468	0.8811	0.0408	0.0005	0.0000

fall spawn contributed significantly to the local population during this study, since no recruits or juveniles were seen during the summer. Additionally, assessment of the size frequency distribution of individuals collected in 1998 (Fig. 2) showed no discernible multiple modes in the distribution. As such, it appears that a single year cohort dominates this population, with few individuals surviving two years. Although large adults did survive the winter of 1998–99 (Table 2, May 1999)

The recent sporadic occurrence of bay scallops in the inland bays of New Jersey might be attributed to one of two scenarios. First, these pulses of adults could be traced to an occasional successful reproductive event by a small resident population. Alternatively, these ephemeral populations may represent a chance recruitment of allochthonous larvae from more stable populations located north or south of New Jersey. These scenarios have different consequences with respect to genetic composition of the New Jersey scallops. The expectation under the first hypothesis is that the New Jersey scallops would be genetically distinct from scallops collected from adjacent populations. While it is true that the New Jersey population is not likely to have been isolated for sufficient time for major genetic divergence to have occurred, the presumed frequent bottlenecking of the population suggested by the absence of detectable numbers in most years would likely have resulted in genetic drift. Under the second hypothesis of episodic recruitment from adjacent populations, the expectation is that the New Jersey scallops would genetically resemble either *A. i. irradians* to the north or *A. i. concentricus* to the south.

The results of the statistical analysis clearly support a closer relationship of the New Jersey scallops with the populations sampled in North Carolina, despite the notable genetic distance between these samples (Table 3). This association is supported by the conventional interpretation of morphological data, which originally described the southern sub-species from type specimens collected from New Jersey. The genetic differentiation between New Jersey and North Carolina may be indicative of some restriction of genetic exchange, although the data presented here are insufficient to conclude that these populations are isolated from one another. The short larval duration of *A. irradians* (10–14 days) makes the probability of direct transport of larvae from North Carolina unlikely. While scallops are occasionally found in the inland bays of Maryland and Virginia, the ephemeral nature and low densities of these populations make them poor candidates as a source for larval export. The absence of evidence to support long distance larval transport in bay scallops is not uncommon. Arnold et al. (1998) found little evidence to support significant larval transport from "high" density populations (St. Joseph's Bay and Steinhatchee) in northwest Florida to "low" density populations further to the south (Crystal River and Anclote). Other studies in the sounds of North Carolina and the Peconic Bay system in New York show a similar lack of recruitment in areas decimated by toxic algal events from adjacent unaffected areas over much smaller spatial scales (Peterson and Summerson 1992, Wenczel et al. 1993). As such, it is likely that the New Jersey population of bay scallops is a resident, albeit cryptic one.

TABLE 4.

Hierarchical analysis of variance on the matrix of distances between scallop mtDNA haplotypes.

	Observed partition			
Variance component	Variance	% Total	p	ϕ statistics
Among regions	0.00553	59.42	<0.001	$\phi_{ct} = 0.549$
Among populations within regions	0.00017	1.79	0.022	$\phi_{sc} = 0.044$
Within populations	0.00361	38.79	<0.001	$\phi_{st} = 0.612$

Population samples were grouped into three regions (New York; New Jersey and North Carolina; Florida). ϕ statistics measure haplotypic correlations and are analogous to the hierarchical *F*-statistics of Cockerham (1969, 1973). *P* values indicate the probability of finding a more extreme variance component and ϕ-statistic than that observed by chance alone.

Assessment of recruitment habitat provided novel insight into the ecology of New Jersey bay scallops. During 1998, juvenile bay scallops were observed in the field during October. While this event alone was not unexpected, the fact that the two recruiting juveniles (shell height 6 and 12 mm) were both attached to adults via byssal threads is intriguing. This behavior was most likely their response to the lack of above ground eelgrass biomass (Fig. 4). This behavior has been observed for Antarctic scallops, *Adamussium colbecki* (Berkman 1988), but observations of this occurring for *A. irradians* have not been recorded. Clearly, for an organism that is so intimately tied to seagrass as primary habitat, the loss of eelgrass signaled a significant loss of recruitment habitat. The loss of eelgrass also may have played a significant role in over-winter mortality. Field collections during November 1998 indicated that substantial passive burial of adults was occurring. Specifically, adults were frequently located within a recessed pit approximately 5–8 cm deep or adjacent to one with sediment fouling apparent on the dorsal shell. Although these observations were not quantified during this period, they suggest that sediment burial was occurring and may have had significant impacts on winter survival. Tettelbach et al. (1990) showed significant winter mortality due to burial for bay scallops in New York, and our observations of burial in 1998 and lack of adults in 1999 correspond to this trend.

Eelgrass not only serves as a primary recruitment habitat for bay scallops, but the beds also alter water velocity, dampen wave energy, and stabilize sediments (Fonseca et al. 1982, Fonseca & Fisher 1986). Consequently, the loss of eelgrass during the summer and fall of 1998 (Fig. 4) may have had direct effects on the abundance of settling bay scallops and also indirect effects on the population, in that the lack of sediment stability may have led to increased winter mortality through burial. These losses in eelgrass biomass correspond to the reduction in bay scallop population density during 1999 (Table 1), but not their elimination from the system.

Since the turn of the century, seagrasses have undergone dramatic declines worldwide due to both natural and anthropogenic sources (Phillips 1982, Cambridge et al. 1986, Robblee et al. 1991). Over the last 25 years, several studies have investigated the distribution of seagrass from coastal New Jersey and have shown significant declines in total coverage (R. Lathrop pers. comm., Bologna unpubl. data). These declines parallel the loss of the commercial and recreational fishery for bay scallops in New Jersey as well (Ford 1997). If continued loss of *Zostera marina* habitat occurs, it may severely limit this population in the future.

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EFFECT OF MICROALGAE PROTEIN ON THE GONAD DEVELOPMENT AND PHYSIOLOGICAL PARAMETERS FOR THE SCALLOP *ARGOPECTEN PURPURATUS* (LAMARCK, 1819)

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ABSTRACT We scanned the effects of food quality on gonadal development and physiological parameters (feeding rate, absorption efficiency, oxygen consumption rate, and excretion rate) for the Chilean scallop, *Argopecten purpuratus*. Scallops were fed microalgal diets with three different protein levels, named low (L), normal (N) and high protein (H), or subjected to starvation (C). In addition, scallops were conditioned to environmental temperature in every season of the year and in the winter two temperatures were tested (field temperature of 10 C and 15 C). The gonads of the groups receiving the normal and high protein diets matured during the conditioning experiments and responded to spawning stimuli. The gonads of scallops fed on the low protein diet did not ripen and could not be stimulated to spawn at the end of the experiments. A maximum fecundity rate and massive spawning, when subjected to spawning stimuli, was obtained in scallops fed a high protein diet in the autumn experiment. Scallops fed a high protein diet increased filtration rate, absorption efficiency, and showed highest scope for growth (SFG) and net reproductive efficiency (K2) values. Medium values of filtration rate, absorption efficiency, SFG and K2 were observed in the scallops fed on a normal protein diet. Scallops fed on a low protein diet had the highest oxygen consumption, lowest filtration rate, decreased absorption efficiency and negative SFG and K2 values. Considering that the low protein diet was rich in carbohydrates, the imbalance of low protein to energy (P/E) could have caused poor protein synthesis when fulfilling energy demands resulting in reduced gonadal growth. However, the poor physiological condition of scallops fed the low protein diet was still better than for starved scallops. The positive SFG observed in the winter at 15 C with the high protein diet showed that the Chilean scallop could be conditioned in the winter by both increased temperature and dietary protein. We propose that physiological responses through reproductive conditioning of the Chilean scallop may be modulated by the protein content of the diet and by the relation P/E of the microalgae. In addition, the increase of protein level of the microalgae improves the SFG and fecundity of scallop broodstock.

KEY WORDS: *Argopecten purpuratus*, mollusc nutrition, physiology, protein requirement, reproduction, scallop

INTRODUCTION

The Chilean scallop, *Argopecten purpuratus*, is being cultured in experimental and commercial hatcheries in the north and south of Chile; and most often larvae are obtained from induced spawning of long-line cultivated adult scallops, without laboratory conditioning (DiSalvo et al. 1984, Uriarte et al. 1996a). Martínez et al. (1999a) provided some data on the physiological aspects of maturation and the importance of nutrition during gonadal development which has been studied by Coutteau et al. (1996), Farías et al. (1997), Marrit et al. (1999), Martínez et al. (1999b).

The biochemical composition of microalgal cells can be varied by altering the nitrogen concentration of the culture medium (Wikfors et al. 1984, Utting 1986, Uriarte et al. 1993). The biochemical modification of the microalgal cultures is an important tool not only for research on the nutritional requirements of filtering organisms, but also for determining the efficient use of microalgae during phases of high consumption for the postlarvae stage and during reproductive conditioning (Uriarte & Farías 1999). Utting (1986) found that oyster larvae grew better when fed carbohydrate-enriched microalgae, while oyster spat grew better on protein-enriched microalgae. Farías et al. (1997) found that the fecundity of adult scallops increases when fed protein-enriched microalgae. Thus, alteration of the nutritional media could be an effective way to control the biochemical quality of microalgae and improve efficiency of production by hatchery (Uriarte & Farías 1995, 1999).

The present study evaluates the effect of biochemically manipulated microalgae on the physiology and gonad development of adult *Argopecten purpuratus* which were introduced in the south of Chile where no natural populations exist.

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MATERIALS AND METHODS

Microalgae of Different Protein Level

We manipulated cultures of *Isochrysis aff. galbana* (clon T-Iso) and *Chaetoceros neogracile*, using the method described by Uriarte et al. (1993) with the modifications of Uriarte & Farías (1999). Three strains pre-conditioned for two years at the different nitrogen levels (high protein, normal protein and low protein) were selected for the bulk production of algae for our experiment on the reproductive conditioning of *Argopecten purpuratus*. As all the cultures were grown using the same light, temperature and aeration conditions, the nitrogen content in the media was the only factor that varied. The microalgae of each species were harvested daily during their exponential growth phase. Three diet treatments were prepared by a mixture of both species in a proportion 1:1 for each protein level: high protein, normal protein and low protein. A fourth treatment was, starving of scallops during the entire conditioning period.

Conditioning Experiments

The scallops were collected from the long-line of the Laboratorio Biológico Pesquero de Putemún (IFOP, Region X, 42°25′S). The scallops were 22 to 30 months old.

The experiments were run during five periods: spring 1995, autumn 1996, winter 1996, spring 1997, summer 1997, at temperatures of 15, 13, 10, 16, and 15°C respectively, corresponding to the average field temperatures.

Scallops were measured, weighed and induced to spawn just before each experiment. Gonad maturation was assessed using a visual index (Mason 1983). During the eight weeks of the experiment, one scallop per tank of 8 L was suspended in recirculated

and filtered (0.5 μm) seawater. The exceptions were the first and last experiment both during springtime, where tanks of 250 L with 15 tagged scallops were employed. In each test, the water was changed and the tanks and tubes cleaned every second day.

The spring 1995 experiment was aimed to detect changes in the biochemical composition of scallop tissues and to determine the optimal food ration for reproductive conditioning. Scallops were fed with two diets: low protein (L) and normal protein (N). There was one tank of 250 L per diet, each tagged scallop in the suspended sieve was considered a pseudoreplicate.

The autumn 1996 experiment examined the effect of three diets and starvation. There were three tanks for each treatment randomly distributed. The diets had three different qualities of microalgae: high (H), low (L) and normal (N) protein level.

The winter 1996 experiment took place when the field temperature was 10°C and the study aimed at the combined effect of temperature and diet. Two temperatures were tested: 10 and 15°C in combination with high and normal protein diets and starvation, keeping three replicates randomly distributed, for each diet/temperature combination.

The spring 1997 experiment evaluated the effect of high and normal protein diets, the pseudoreplicates were 15 tagged scallops for each diet suspended in a sieve inside of 250 L tank with recirculated water.

The summer 1997 experiment evaluated the effect of high, low and normal protein diets, with three tanks for each treatment, randomly distributed.

The microalgal diets were continually delivered to the broodstock with a peristaltic pump. The daily rations were based on the results of the spring 1995 experiment.

Biochemical Analysis

At the end of the first experiment, we made biochemical analyses of gonad, digestive gland and muscle of scallops, as these body components are likely sites of energy storage for subsequent use in gonad maturation (Martínez 1991, Uriarte et al. 1996b).

During the conditioning period of the autumn experiment the biochemical analysis of each microalgae species and quality was run. Samples of known concentration and volume of microalgae were filtered on pre-ashed and pre-weighed Whatman GF/C filters using a vacuum system (pressure less than 5 in. Hg). The filters were dried to constant weight at 40°C and subsequently frozen at −20°C until analyzed. The biochemical characterization of the various mixtures of microalgae was estimated from biochemical data on individual microalgal species.

The biochemical analysis were carried out using the methods cited by Farías et al. (1998) using bovine gamma-globulin, cholesterol and glycogen of oyster as standards for protein, lipid and carbohydrate, respectively. The energetic content of the diet was calculated based on the energetic equivalents for protein, lipid and carbohydrates of 23.5, 39.37 and 17.08 J mg^{-1}, respectively, reported by Paine (1971).

Physiological Analysis

Toward the end of each conditioning period, physiological evaluations were carried out on the scallops. To determine the clearance rate (CR) a closed system was used (Widdows 1985). Each experimental scallop was placed in a 1-L glass beaker, highly aerated and with an initial concentration of 10 algae-cells μl^{-1}. The reduction in particle concentration was measured in samples of 10 ml every 15 min for 1 h. An additional container with the same characteristics but with no scallop served as the control. The particles were counted after centrifugation and removal of 9.5 ml of supernatant. The experimental scallops were left undisturbed for at least 24 h before the clearance rate measurements started. CR (l h^{-1}) was calculated by the exponential regression:

$$C_t = C_0 \times e^{(-(m n M)+a)\times t}$$

where C_t is algae concentration after time t. C_0 is initial cell concentration, m is filtering rate of a single animal, n is number of scallops, M is volume of suspension, a = rate at which particles settle out of suspension in the control beaker, calculated from:

$$C'_t = C'_0 \times e^{-at}$$

The ingestion rate was estimated as the product of the clearance rate (l h^{-1}) and the cell concentration (mg L^{-1}). The feeding rate (FR) was estimated as the product of the ingestion rate (mg h^{-1}) and the energy of microalgae mixture (J mg^{-1}). The absorption efficiency (AE) was measured for each individual using the method found in Conover (1966), on the basis of fecal organic content and of food consumed during the previous 8 h. The absorption rate (AR) was estimated as the product of the feeding rate (J mg^{-1}) and absorption efficiency. The rates of oxygen consumption (VO_2) were measured at the experimental temperature in a 1.5-L closed chamber with a Strathkelvin Polarographic electrode following the methodology of Widdows (1985). The rates of ammonium excretion (VNH_4-N) were determined with the method described by Widdows (1985). Scope for growth (SFG) and net growth efficiency (K2) are reference values for the production of somatic and/or reproductive tissue, depending on the age of the bivalve (Vahl 1981). In this case the scallops were adults subjected to a conditioning regime, then the production was considered only as growth of the gonad tissue. For this reason was used the terms scope for reproductive growth for SFG and net reproductive efficiency for K2. The scope for reproductive growth (SFG), the net reproductive efficiency (K2), and the stress index (O/N ratio) were calculated by incorporating all previous physiological measurements as described by Widdows (1985). At the end of the physiological evaluation, all scallops were weighed and induced to spawn.

At the end of each experiment the body components were separated and dried at 100°C for 72 h prior to weighing. All physiological determinations were then standardized to a mean dry body mass of 4 g (close to an average scallop, 4.06 ± 0.48 g), using the equation of Bayne et al. (1993) and the regression coefficients for filtration rate, oxygen uptake, and excretion rate of Navarro and González (1998) at salinity of 30 g L^{-1}.

Statistical Analysis

The protein and energy content of the diets were compared between the two microalgae species and nitrogen levels using an ANOVA. The diet quality was characterized by the ratio protein/energy (P/E, both measured in Joules) and the total food energy availability per scallop (FE). The physiological rates and the spawning response between treatments were compared statistically using ANOVAs (Sokal & Rohlf 1981). The absorption efficiency values were arcsine transformed, because they were ratios, in accordance with Sokal and Rohlf (1981). Correlation and multiple linear regressions analyses were carried out following $\log_{10}$ transformation of the physiological rates, to reduce the dependence of the sample variance on the mean and to normalize the distribution

of the data (Navarro and Thompson 1996). The variables included in the analyses were temperature, diet quality, FE (J h^{-1}) and protein absorbed (J h^{-1}). Protein absorbed (PA) was estimated as the product of the energy absorbed (J h^{-1}) and the percentage of protein content for each diet quality. Average data were expressed as mean ± standard error.

RESULTS

Manipulated Microalgae

The protein content of the microalgae was significantly different between the qualities generated. *C. neogracile* showed a higher protein content than T-*Isochrysis* whereas the various quality cultures showed a decreasing protein content (F_{spp} = 11.53, d.f.= 1;18, p = 0.005; F_N = 30.82, d.f. = 2; 18, p < 0.0001) as follows: H > N > L (p = 0.05). The energetic contents showed that there were no significant differences between the microalgal species used. The mixture of both microalgae (Table 1) was characterized by significant differences on the protein content (F = 35.18, d.f. = 2, 7, p = 0.001), carbohydrate content (F = 36.73, d.f. = 2, 7, p = 0.001) and ratio P/E (F = 15.56, d.f. = 2, 7, p = 0.007) between qualities. The total energy, dry mass and organic matter of mixtures were similar for the three levels of protein, with averages values of: 0.94 ± 0.04 J per million cells, 44.5 ± 5.9 µg per million cells and 67.3 ± 4.0%, respectively.

Scallop Conditioning

During the spring 1995 experiment, a daily ration of 2.5 × 10^9 cells day^{-1} $scallop^{-1}$ of both the low or normal protein diets was sufficient to stimulate the ripening of gonads without producing pseudofeces. Therefore, this ration value was used as constant for subsequent experiments. The absorption efficiency values did not differ significantly between diets (Table 2), the mean absorption efficiency being 57.33% (±2.16). The Mason Index did not show significant difference in the appearance of gonads between diets. The scallops fed on the L diet had a lower gonad index.

Protein levels of scallop tissues did not differ between diets after conditioning (Table 3). However the differences were significant between tissues: 14.3 ± 2.5, 79.9 ± 3.4 and 90.3 ± 3.4% of dry weight of digestive gland, gonad and muscle, respectively. Lipid values of scallop tissues varied significantly between diets (Table 3), with maximum values for diet L (15.6% ± 1.27 of dry weight). Lipid levels between tissues were significantly different (p = 0.05) (Table 3), with the highest value for the gonad (17.6% ± 1.6 of dry weight) followed by the muscle (12.5%) and digestive gland (9.8%). The carbohydrate levels did not show differences between diets. The values varied significantly between tissues (Table 3), with low values in gonads and muscles (3.5 and 2.4% of dry weight, respectively) and a high level in the digestive gland (26.8% ± 0.9 of dry weight). Thus, only tissue lipids varied between diets.

In the autumn experiment, the clearance rate differed significantly between treatments (Table 4, F = 53.47; d.f. = 3, 8; p = 0.001). The lowest rate was observed in the starved scallops, the highest rate in the scallops fed on the H diet and there were no significant differences between the N and L diets. Oxygen uptake (Table 4) showed no significant difference between treatments. The mean VO_2 was 2.50 ml O_2 h^{-1} (±0.29), with a maximum rate (p = 0.06) seen in the scallops receiving diet L and a minimum observed in scallops fed on the N diet. The VNH_4 – N (Table 4) did not show significant differences between treatments although the highest value was observed in the starved scallops. The mean excretion rate was 193.58 (±26.46) mg NH_4-N h^{-1}. Absorption efficiency values was significantly different between diets (Table 5, F = 9.91; d.f. = 2, 17; p = 0.002), with the highest efficiency in the diet H (66.4%). No significant differences were observed between the diets N and L. The starved scallops did not show a positive absorption. Scope for growth (SFG) was significantly higher in the broodstock maintained on the N and H diets (Table 5), (F = 14.44, d.f. = 3, 8; p = 0.007), making available 23.2 and 141.3 J h^{-1} of energy, respectively, for the production of new tissues and gametes. The starved scallops and those fed the diet L showed negative SFG. Net growth efficiency (K2) was 58% in diet N, and 72% in diet H. The negative values indicated that the starved scallops and the ones maintained on diet L did not produce new tissues (Table 5), F = 16.58; d.f. = 3, 8; p = 0.005). The O/N ratio did not show significant differences between diets, although the higher values for O/N, that it means less stress, were observed with low protein diet associated to high VO_2. When induced, the scallops fed on the N and H diets spawned completely, whereas the diet L scallops were observed to release sperm only. The mean fertility was 2.8 × 10^6 (±1.1 × 10^6) eggs $scallop^{-1}$

TABLE 1.

Mean values (± the standard error) for microalgal quality after manipulation of nitrogen in the culture media.

	H	N	L
Protein (µg/10^6 cells)	7.70 ± 0.63	5.83 ± 0.22	1.72 ± 0.46
Lipid (µg/10^6 cells)	18.22 ± 1.82	17.62 ± 1.34	23.11 ± 1.15
Carbohydrate (µg/10^6 cells)	1.82 ± 0.14	2.97 ± 0.37	5.55 ± 0.33
Energy (J/10^6 cells)	0.93 ± 0.06	0.88 ± 0.06	1.04 ± 0.03
P/E	0.20 ± 0.03	0.16 ± 0.01	0.04 ± 0.01
Dry weight (µg/10^6 cells)	51.21 ± 9.86	39.08 ± 11.12	42.72 ± 14.65
Organic matter	0.77 ± 0.04	0.62 ± 0.03	0.59 ± 0.10

TABLE 2.

***Argopecten purpuratus*. Evaluation of reproductive conditioning with normal and low protein diet made by mixing N-low or N-normal cultures of *C. neogracile* and *I. galbana*. Mean values (± the standard error) are given. There were 10 to 15 replicates per treatment.**

Diet	Absorption EFF (%)	Final weight (g)	Mason index
Normal protein (N)	59.9 ± 3.2	63.8 ± 1.0	4.3 ± 0.2
Low protein (L)	53.7 ± 3.0	63.6 ± 1.4	3.7 ± 0.2

TABLE 3.

Factorial ANOVA statistics of the biochemical composition of tissues of scallops fed on L and N diet. Data of percentage values of dry weight were transformed by arcsin.

		Protein		Lipid		Carbohydrate	
Factor	d.f.	F	p	F	p	F	p
Diet	1	0.16	0.7	1.43	0.02	0.14	0.7
Tissue	2	99.95	0.00001	6.42	0.005	347.69	0.00001

TABLE 4.

***Argopecten purpuratus*. Physiological rates of standard scallops of 4 g dry tissue weight conditioned with experimental diets.**

Diet	Clearance rate (l h^{-1})	Oxygen uptake (ml O_2 h^{-1})	Ammonia excretion (µg NH_4 – N h^{-1})
Autumn			
C	0.91 ± 0.01	2.69 ± 0.15	273.41 ± 40.97
H	34.76 ± 1.36	2.39 ± 0.12	181.07 ± 47.26
L	2.31 ± 0.63	3.27 ± 0.69	171.59 ± 40.41
N	6.08 ± 1.54	0.95 ± 0.14	166.54 ± 21.74
Winter			
Temperature 10 C			
C	7.61 ± 0.00	—	15.93 ± 00.00
H	15.23 ± 1.46	1.42 ± 0.49	138.38 ± 69.07
N	8.18 ± 0.33	1.46 ± 0.23	180.41 ± 60.25
Temperature 15 C			
C	1.39 ± 0.00	0.24 ± 0.00	387.26 ± 00.00
H	8.69 ± 0.44	1.23 ± 0.24	484.86 ± 148.66
N	6.83 ± 2.22	1.38 ± 0.11	216.24 ± 87.64
Summer			
H	17.94 ± 3.05	1.65 ± 0.10	450.03 ± 58.02
L	12.70 ± 2.68	2.49 ± 0.45	326.44 ± 8.13
N	19.21 ± 2.28	1.80 ± 0.29	464.89 ± 11.57
Spring			
H	3.29 ± 0.59	2.76 ± 0.39	448.59 ± 35.17
N	2.81 ± 0.67	2.98 ± 0.39	560.89 ± 59.81

H, L and N refers to high, low and normal protein algae, respectively. C refers to the treatment of starvation.

for diet N and 13×10^6 ($\pm 0.5 \times 10^6$) eggs scallop^{-1} for diet H. Egg diameter was 61.3 mm (±0.4) with no significant differences between the treatments. The eggs were viable and developed into scallop embryos.

There were no significant differences in clearance rate between temperatures or between diets during the winter experiment. Higher clearance rate values were characteristic for the H diets at both temperatures. The lowest clearance rate was observed in the starved scallops. Oxygen uptake did not present significant differences between temperatures but there was a significant effect between the diet (Table 4, $F_{diet} = 5.52$, d.f. = 2,11; $p = 0.04$). The minimum value for VO_2 was observed in the starved scallops. The results of VNH_4-N showed no significant difference between temperatures or diets and had a mean value of 237.84 (±57.61) mg NH_4-N h^{-1} (Table 4). Absorption efficiency values was also not affected by temperature and diet, ranging from 34.8% in diet N at 10°C to 48.2% in diet H at 15°C (Table 7). The starved scallops did not show absorption. The calculated SFG and K2 did not differ between temperatures and diets (Table 5). Higher values were observed for diet H. The highest value of SFG and K2 were observed with diet H at 10°C. The scallops fed on N and H diets at 15°C spawned when induced, whereas the scallops at 10°C did not release sperm or eggs. The eggs obtained at 15 C appeared coagulated and were not viable.

In the summer 1997 experiment, values of clearance rate did not differ between treatments (Table 4), although clearance rate was lowest in the L diet. There were no significant differences in VO_2 between the diets (Table 4). The tendency of Oxygen uptake was the same as in the above experiments, with maximum values for the scallops fed on diet L. Results of VNH_4-N indicated no significant differences between diets in spite of apparently low value for the L diet (Table 4). Absorption efficiency values were the lowest during the study and did not vary with the diet (Table 5). SFG did not differ between the treatments (Table 5) and was negative for all diets with values as low as −24.6 J h^{-1} (L diet). K2 as well was negative (Table 5), ranging between −1.6 and −1.1 for scallops fed on diets L and H, respectively. The O/N stress index tended to higher values with a low protein diet, showing a less stress condition of scallops, however only the scallops fed on N and H diets responded to the spawning induction. Scallops fed on the diet L did not release sperm or eggs and died 24 h from the effects of stimulating to spawn.

In springtime, the last experiment of 1997 revealed no significant differences for clearance rate between treatments (Table 4), although the highest value was observed for diet H. VO_2 was similar between the diets (Table 4). Excretion rate in *A. purpuratus* did not differ between diets N and H. The mean value of VNH_4-N was 504.72 (±38.87) µg NH4-N h^{-1} (Table 4). Absorption efficiency values were not affected by the diets N and H (Table 5) and had a mean value of 52.7 (±3.5) percent. SFG was negative for both diets (Table 5) with a mean value of −54.6 J h^{-1} and no significant difference between treatments. Similarly, K2 was negative (Table 5) in both diets and was associated with a low clearance rate and a high oxygen uptake. The diets had a similar O/N ratio with a mean value of 7.6 (±0.94) (Table 5). During the spawning induction, the scallops did not respond to the stimuli and died after 24 h.

Integrated Results

Clearance rates showed significant differences between seasons ($F_{season} = 8.735$; d.f. = 3,48; $p = 0.0001$) with a minimum clearance rate of 1.61 l h^{-1} (SE = 3.53, n = 10) during winter and a maximum clearance rate of 12.13 l h^{-1} (SE = 2.12, n = 25) during summer. There were significant differences between diets ($F_{diet} = 5.510$; d.f. = 3, 48, $p = 0.0025$). The minimum CR of 0.93 l h^{-1} (SE = 3.11, n = 4) was observed in the starved scallops while the maximum rate of 10.83 l h^{-1} (SE = 1.49, n = 21) was found in diet H. Feeding rate (FR) was positively correlated with the scallop weight, ratio protein/energy (P/E) of diet and seasonal temperature, while the correlation of FR with absorption rate and protein absorbed was attributed to the autocorrelation of these variables (Table 6). Multiple regression analysis between FR and the experimental variables accounted for 45% of the variation (Table 7). Dry mass of the scallops alone explained 20% of the variation.

Absorption efficiency varied significantly with seasons ($F_{season} = 15.69$; D.F. = 1, 153; $p = 0.00001$), with maximum values in autumn (68.25%, SE = 4.26, n = 18) and minimum values in summer (23.18%, SE = 4.26, n = 18). No significant differences were observed between the diets. The starved scallops showed a negative absorption. The overall annual mean value for absorption efficiency, independent from the diets, was 50.52% (SE = 1.77, n = 160). The absorption rate (J h^{-1}) correlated with many of the nutritive variables, and showed an especially close correlation with the energy and protein level of the diet (Table 6). Food energy availability and ratio protein/energy (P/E) explained 59% of the variation in absorption rate (Table 7).

Oxygen uptake showed significant differences between seasons ($F = 8.54$, d.f. = 3,51, $p = 0.0001$). The highest value, of 2.68 ml O_2 h^{-1} (SE = 0.27, n = 14), was in spring and the lowest, 1.30

TABLE 5.

***Argopecten purpuratus*. Scope for growth for a standard scallop of 4 g dry tissue weight conditioned with diets.**

Diet	Feeding rate (J h^{-1})	Absorption efficiency (%)	Absorption rate (J h^{-1})	Rate of Oxygen consumption (J h^{-1})	Excretion rate (J h^{-1})	SFG (j h^{-1})	K2	O:N
Autumn								
C		—		54.7 ± 3.0	6.8 ± 1.0	−61.5 ± 2.7		13.13 ± 2.81
H	318.1 ± 12.5	66.4 ± 3.8	195.1 ± 26.1	48.6 ± 2.5	4.5 ± 1.2	141.3 ± 22.3	0.72 ± 0.20	16.54 ± 3.22
L	24.2 ± 6.5	59.3 ± 3.6	19.8 ± 5.7	66.5 ± 14.1	4.3 ± 1.0	−50.9 ± 8.0	0.58 ± 0.19	27.68 ± 7.90
N	58.4 ± 8.0	57.5 ± 3.8	35.1 ± 4.9	19.3 ± 2.8	4.1 ± 0.5	23.2 ± 16.5	−0.21 ± 0.64	8.05 ± 2.27
Winter								
Temp: 10 C								
C	72.9 ± 0.0	—		−3.3 ± 0.0	0.4 ± 0.0	−7.5 ± 7.1		
H	139.3 ± 13.4	45.6 ± 4.7	61.9 ± 0.8	28.9 ± 10.0	3.4 ± 1.7	16.0 ± 11.7	0.48 ± 0.13	34.5 ± 21.7
N	78.5 ± 3.2	34.8 ± 3.8	27.7 ± 1.8	29.8 ± 4.7	4.5 ± 1.5	−7.3 ± 8.6	−0.28 ± 0.31	11.7 ± 2.3
Temp: 15 C								
C	13.2 ± 0.0	—		4.9 ± 0.0	9.7 ± 0.0	−14.6 ± 0.0		
H	79.5 ± 4.0	48.2 ± 5.1	39.5 ± 5.8	25.0 ± 5.0	12.1 ± 3.7	2.5 ± 14.7	−0.05 ± 0.37	3.4 ± 0.4
N	65.5 ± 21.3	40.5 ± 3.1	29.8 ± 9.7	28.1 ± 2.3	5.4 ± 2.2	−8.0 ± 12.5	−0.88 ± 0.78	5.4 ± 0.3
Summer								
H	33.6 ± 2.1	20.1 ± 4.4	31.9 ± 6.2	33.6 ± 2.1	11.2 ± 1.4	−12.9 ± 7.2	−1.1 ± 0.5	5.3 ± 0.9
L	50.6 ± 9.1	28.6 ± 6.5	34.1 ± 7.8	50.7 ± 9.1	8.1 ± 0.9	−24.6 ± 10.5	−1.6 ± 0.6	11.4 ± 2.8
N	36.7 ± 5.8	19.5 ± 3.8	34.3 ± 6.9	36.7 ± 5.8	11.6 ± 1.4	−13.2 ± 7.1	−1.2 ± 0.7	6.2 ± 1.2
Spring								
H	30.1 ± 5.4	51.9 ± 4.7	16.9 ± 2.8	56.2 ± 8.0	11.2 ± 0.9	−50.4 ± 10.4	−5.3 ± 2.6	7.8 ± 1.0
N	26.9 ± 6.5	53.4 ± 3.8	15.9 ± 4.3	60.7 ± 7.9	13.9 ± 1.4	−58.8 ± 8.4	−12.9 ± 6.2	7.5 ± 1.5

H, L and N. C refers to the starved scallops.

ml O_2 h^{-1} (SE = 0.35, n = 12), in winter. Oxygen consumption rates tended to vary between diets (p = 0.06). Scallops receiving diet L tended to consume most oxygen (2.54 ml O_2 h^{-1}, SE = 0.28, n = 11) and the starved scallops least (1.47 ml O_2 h^{-1}, SE = 0.43, n = 4). VO_2 was positively correlated with the seasonal temperature and food energy availability (Table 6), and negatively correlated with the total food cells in the tanks (FT). The multiple regression models explained 31% of total variation in oxygen uptake (Table 7), with food energy availability accounting for 14%.

The ammonium excretion rates showed seasonal differences (F_{season} = 3.099, d.f. = 3,55, p = 0.03) with a maximum value of 478.19 mg NH_4-N h^{-1} (SE = 49.45, n = 14) in spring time and a minimum of 288.63 mg NH_4-N h^{-1} (SE = 64.27, n = 11) in autumn. The ammonium excretion rates did not vary with diets. VNH_4-N in *A. purpuratus* was highly correlated with the experimental temperature, the seasonal temperature and the food cells in the tanks (Table 6). The best multiple regression model for VNH_4-N showed that 48.5% of the variation was explained by experimental temperature, ratio protein/energy (P/E) and feeding rate, whereas experimental temperature alone accounted for 41% of the variation (Table 7).

The reproductive SFG (Fig. 1) varied between seasons (F_{season} = 26.27, d.f. = 0.3, 34, p < 0.0000001) and between diets (F_{diet} = 17.24, d.f. = 1, 34, p = 0.0002). SFG was negative during spring and summer, close to zero during winter and above zero during autumn SFG. There were significantly higher values of SFG in scallops fed on diet H. The seasonal difference was most conspicuous in starved *A. purpuratus* (Fig.2) (F_{season} = 6.86, d.f. = 1, 9, p = 0.03). The SFG was always negative, but ranging from values as low as −62.78 J h^{-1} (±17.05) in autumn to values very close to zero in winter. SFG for three N-diets plotted (Fig.3). During summer, SFG was not influenced by the diet. In contrast, SFG was affected by the diet in autumn, suggesting that there was a strong positive relationship between SFG and ratio protein/energy (P/E) in this season ($F_{season\ P/E}$ = 18.29, d.f. = 2, 26, p = 0.0000009). Scope for reproductive growth was correlated with the nutritive variables, protein absorbed and total food availability, and with two physiological variables, feeding rate (FR) and VO_2 (Table 6). Multiple regression analysis showed that 68% of the total variance of SFG was explained by protein absorbed, food availability, VO_2 and FR, whereby protein absorbed alone accounted for 23% (Table 7).

The net reproductive efficiency (K2) was highly correlated with the seasonal temperature, food energy availability, and absorption rate (Table 6). Food energy availability alone explained 33% of the variation and when the seasonal temperature, absorption rate, FR and the O/N ratio were included, 86% of the variation was explained (Table 7).

The O/N stress index was negatively correlated with the experimental temperature and food energy availability (Table 6). The variation of O/N was best explained by the model which included the seasonal and the experimental temperature, food energy availability and ratio protein/energy (P/E) as independent variables, explaining as such only 27% of the variation (Table 7).

DISCUSSION

The experiments on conditioning *A. purpuratus* showed that scallops fed on the high protein diet, had both the highest scope for reproductive growth (SFG) and net reproductive efficiency (K2) (parameters considered as indexes of success of reproductive conditioning, because the production was not detected as somatic growth but as gonadic growth), followed by the scallops conditioned on the normal protein diet. The scallops fed on the low protein diet or starved had the lowest SFG and K2 values.

TABLE 6.

***Argopecten purpuratus*. Correlation coefficients between physiological and nutritional variables.**

	FR	AR	VO_2	VN-NH_4	SFG	K_2	O/N	Season	ExpT	DWT	P/E	PA	FE	FT	FS
FR	1.00	0.50	−0.19	−0.10	0.61	−0.31	−0.06	0.24	0.18	−0.46	0.25	0.47	0.04	0.02	−0.01
	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)
		***			***	*				***	*	***			
AR		1.00	0.17	−0.01	0.18	−0.33	0.13	0.24	0.13	−0.16	0.49	0.25	0.65	0.07	0.51
		(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)
						*					***		***		***
VO_2			1.00	0.08	−0.58	−0.27	0.64	0.29	0.05	−0.08	−0.04	−0.02	0.27	−0.32	0.12
			(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)
					***	*	***	*					*	*	
VN-NH_4				1.00	−0.22	0.08	−0.71	0.37	0.55	−0.20	0.15	−0.20	−0.14	−0.48	−0.16
					(56)	(0.56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)
							***	**	***					***	
SFG					1.00	−0.14	−0.25	−0.13	−0.09	0.01	0.15	0.51	−0.01	0.29	0.02
					(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)
												***		*	
K2						1.00	−0.25	−0.59	−0.22	0.14	−0.03	−0.36	−0.56	0.23	−0.53
						(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)
								***				**	***		***
ON							1.00	0.08	−0.39	0.09	−0.14	0.14	0.30	0.14	0.21
							(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)
									**				*		

* $p < 0.05$
** $p < 0.001$
*** $p < 0.0001$

Number of cases in parentheses. FR: feeding absorption rate; VO_2 oxygen consumption rate; VN-NH_4: ammonia excretion rate; SFG: scope for growth; K_2: net growth efficiency; O/N: stress index; Season: seasonal; ExpT: experimental temperature; DWT: scallop dry weight; P/E: quality diet index; PA: protein absorbed; FE: food energy availability per scallop; FT: total food cells in the tanks; cells availability per scallop.

The clearance rate ($l\ h^{-1}$) and the absorption efficiency values followed the same pattern as described in the preceding paragraph, highest values on diet H, medium on diet N and the lowest values in the scallops fed on diet L. Our results suggest that improvement of reproductive conditioning on *A. purpuratus* is the product of the combined effect of high clearance rate and increased absorption efficiency values. The same pattern to maximize the net energy in bivalves when food quality increases is described by Navarro et al. (1994).

The respiration rates (VO_2) did not follow the same pattern of SFG or K2 in relation to the protein content of the diets. The lowest respiration rates were for the scallops fed a normal protein diet, and reflected their low level of activity compared to the scallops in the other treatments. Shumway et al. (1988) found that metabolic rate is simultaneously affected by food, temperature and reproductive stage, and state that it is difficult to separate these effects without a good knowledge of how energy is allocated between somatic growth and gametogenesis. We observed an unexpectedly high VO_2 in the non-fed group in autumn, which might have been caused by the food particles consumed during the clearance rate test the previous day. However, the feeding activity did not provoke an increase in oxygen consumption in other pectinid genera such as *Chlamys*, although an increase was observed in mytilids (Mackay & Shumway 1980).

Excretion rates were not affected by dietary protein levels, contrary to the findings of Hawkins and Bayne (1991). Scallops required more proteins than mussels as previously reported in studies in *Argopecten irradians* by Barber and Blake, (1985) and Epp et al. (1988). The level of energy in the high protein diet (where we

TABLE 7.

***Argopecten purpuratus*. Multiple regression statistics for several physiological variables or physiological index vs. subsets of nutritive variables. See Table 6 for abbreviations.**

Dependent var.	Independent var.	R^2	n	F
FR	DWT	0.20	56	15.21***
	P/E	0.26	56	5.55*
AR	FE	0.46	56	49.07***
	P/E	0.59	56	19.63***
	SeasonT	0.66	56	12.04**
VO_2	FE	0.13	56	10.46**
	FT	0.23	56	6.94*
VNH_4	Exp.T	0.41	56	42.45***
	P/E	0.44	56	3.67
SFG	PA	0.23	56	18.16***
	VO2	0.58	56	43.06***
	FR	0.67	56	13.75**
K2	FE	0.33	56	28.87***
	SeasonT	0.53	56	25.93***
	AR	0.66	56	20.27***
O/N	Exp.T	0.14	54	10.22**
	FE	0.18	54	3.82*
	SeasonT	0.26	54	6.27*

* $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

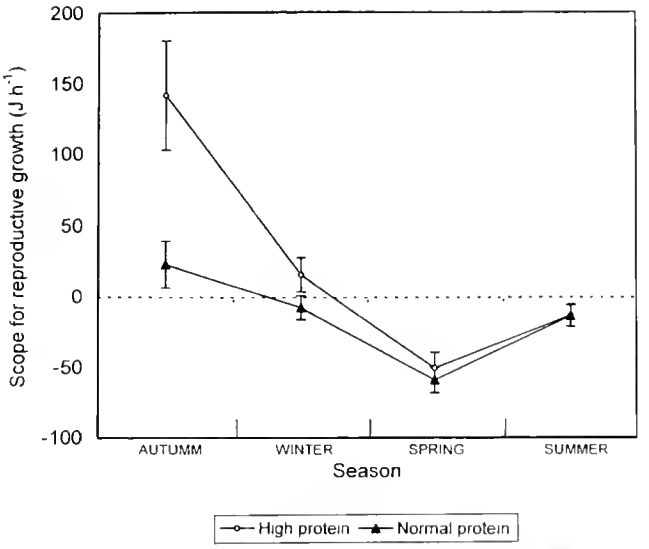

Figure 1. ***Argopecten purpuratus.*** **Seasonal changes in the scope for reproductive growth during conditioning experiments in a Chilean scallop of 4-g dry tissue weight.**

expected a maximum VNH_4-N) could have been enough to use the available protein, producing more gametes and reducing losses due to NH_4-excretion The high VNH_4 observed in the starved scallops suggest the use of protein to meet metabolic demands during prolonged starvation as previously documented in mussels and in the scallop *Argopecten irradians* (Epp et al. 1988, Hawkins & Bayne 1991).

The similarity in egg size for scallops fed high and normal protein diets indicated that the additional protein in the high protein diet is mainly used for the production of a larger number of eggs and not for larger eggs. A similar strategy has been observed for *Placopecten magellanicus*. Scallops found in poor environmental conditions had a reduced fecundity but the quality of the eggs was maintained (Thompson & MacDonald 1990, Napolitano et al. 1992).

The maintenance costs of scallops with a low protein diet exceeded the energy assimilated. Diet L scallops had lower clearance rate and absorption efficiency, as well as a high tendency to form

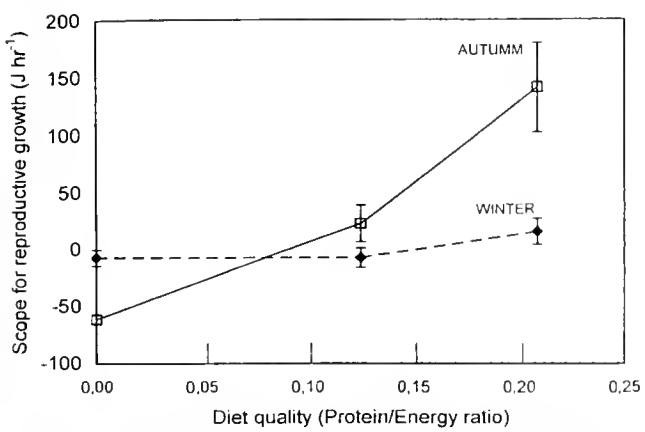

Figure 2. ***Argopecten purpuratus.*** **Comparison between autumm and winter of the effect of diet quality upon the scope for reproductive growth for a standard scallop of 4-g dry tissue weight**

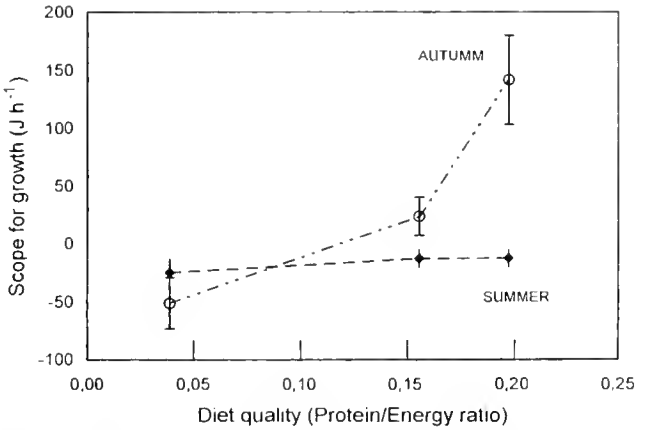

Figure 3. ***Argopecten purpuratus.*** **Comparison between autumm and summer of the effect of diet quality upon the scope for reproductive growth for a standard scallop of 4-g dry tissue weight.**

pseudofeces and higher oxygen uptake. The reduction in clearance rate and absorption efficiency values together with pseudofeces production are feeding responses of bivalves predicted by Navarro et al. (1994) when food quality is poor. We observed a tendency to increase oxygen consumption during oogenesis of *Argopecten irradians* indicating metabolic use of carbohydrate (Barber & Blake, 1985) and in our study could be related to a high degradation of dietary carbohydrates. On the other hand a maximum lipid value observed in the tissues of scallops fed diet L, was not related to the lower gonad index in the same scallops, but could be indicated that a carbohydrate excess of this diet was being converted to lipids which are presumably stored in developing ova. Considering that diet L was very rich in carbohydrates and showed the lowest protein content and the lowest ratio protein/energy (P/E) index, it could have caused a dietary imbalance such as documented for fish, characterized by an excess of dietary energy associated with a decreased protein intake, poor protein synthesis and reduced growth (Young Cho 1987, Tacon 1990). However, the physiological condition of the diet L scallops was still better than that of the starved scallops, and they may be diagnosed as undernourished scallops.

Massive spawnings in the diets H and N were only observed in the autumn. In the other seasons, the gonads were ripe but could not be stimulated to spawn and this was associated with a negative SFG. We propose that the microalgal requirements varied seasonally and 2.5×10^9 cell day^{-1} $scallop^{-1}$ was enough to develop gametes in spring 1995, autumn 1996 and winter 1996, but did not meet energy requirements to mature or to spawn in the other seasons or when diet L was applied. Loss of the relationship between SFG and diet quality in summer could be attributed to insufficient food. This was suggested by the mortality after stress from spawning induction. The negative SFG observed in spring 1997 could have been caused by the change in the culture system. The movement of the scallops was more restricted and they decreased their consumption rate. The mortality after induction of spawning in this experiment was likely associated with the increased requirements to fuel the sudden increase in gonad growth. The positive SFG observed in winter 1996 at 15°C with diet H showed that the Chilean scallop could be conditioned over the winter by increasing temperature and dietary protein level.

The net reproductive efficiency (K2) was the parameter most related to seasonal temperature and its intense effect on VO_2. The

O/N ratio (stress index) was lower than that reported for the Chilean scallop by Navarro and González (1998) and this may be related with the higher reproductive activity of the scallops in our study. The low values of O/N index associated with high excretion rates of reproductive scallops mean that gametogenesis in scallops occurs mainly at the expense of the adductor muscle protein as suggested by Barber and Blake (1985) and Epp et al. (1988).

Low temperature or low energy availability might have caused high stress condition in reproductive adult broodstock conditioned at 10°C in the winter experiment. Similarly, scallops from the field in southern Chile at the end of winter, experienced massive mortalities when they were ripe (Farías et al. 2001). The Chilean scallops in our study are below their normal distributional limit (35° S) and represent second generation individuals originating from broodstock from northern Chile (Region IV, 30°16′S) (Uriarte et al. 1996a). We propose that southern *A. purpuratus* and some populations of *A. irradians* could be nutritionally and climatically stressed, (Barber & Blake 1985) since available food and glycogen reserves are insufficient to support reproduction and maintenance metabolism. In addition, the catastrophic mortalities observed at southern Chile, could be associated with the three main factors that affect the seasonal metabolism of adult scallops (Shumway et al. 1988, Uriarte et al. 1996 b): stress caused by low temperature (<10°C), low food availability, and high energetic demand for gonad ripening.

Martínez et al. (1999 b), showed that microalgae enriched with a mixture of lipids improved the conditioning of *A. purpuratus*. Uriarte and Farías (1995, 1999) have shown that protein content of the microalgal diet affect the physiology and growth of juvenile scallops up to a size of 2 mm, and after this other dietary factors are probably more important. The present work confirms the importance of increasing protein in microalgae to improve the efficiency of massive production in the hatchery. The diet H influenced the SFG and the gonadic growth of scallop *A. purpuratus* by the increase of clearance rate, absorption efficiency values and the improvement of the relationship between oxygen uptake and ammonia excretion. The interaction between dietary protein and lipid enrichment on gonad ripening would be an interesting task for future investigation.

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LITERATURE CITED

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STEPHANOSTOMUM SP. (TREMATODA: ACANTHOCOLPIDAE), THE CAUSE OF "PIMIENTILLA" DISEASE IN CATARINA SCALLOP *ARGOPECTEN VENTRICOSUS (CIRCULARIS)* (SOWERBY II, 1842) IN BAJA CALIFORNIA SUR, MÉXICO

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ABSTRACT We investigated the etiology of a disease of catarina scallop (*Argopecten ventricosus (circularis)*) during an outbreak in Baja California Sur, B.C.S., México, in the summer of 1995. Samples from Bahía Magdalena and Bahía Concepción were studied. Our results indicate that the disease named "pimientilla" is caused by encysted metacercaria of the trematode *Stephanostomum* sp. The melanized spots in the adductor muscle characteristic of the disease are cysts of this trematode. Almost all scallops analyzed had abundant cysts. The characteristics of the matacercariae are described and compared with that of other species of *Stephanostomum*.

KEY WORDS: *Stephanostomum*, metacercaria, catarina scallop, disease

In Baja California Sur (B.C.S.), México, the catarina scallop (*Argopecten ventricosus (circularis)*) fishery is an important economic activity employing about 7,000 families in extracting, processing, and marketing. The production of catarina scallop was 7,000 t of adductor muscle during 1989 and 1990, with more than 4,500 t in 1989 from Bahía Magdalena and Bahía Concepcion (Massó 1996).

In recent years, the catarina scallop fishery has been affected by the collapse of natural stocks and by the presence of some parasites, which caused a decrease in production volumes. During 1978 and 1992 the fishing areas of La Paz and Bahía Concepción, respectively (Fig. 1) suffered a collapse of their natural stocks. Such events were associated with overfishing in La Paz (Baqueiro et al. 1981) and with adverse environmental conditions in Bahía Concepción (Massó 1996). During 1978, the commercial fisheries in Lagunas Guerrero Negro and Ojo de Liebre B.C.S. were closed temporarily by the Mexican Health Ministry. This was necessary because an outbreak of nematodes (*Echinocephalus pseudouncinatus* in the adductor muscle) was detected, which required an evaluation of their effect on human health (Gómez del Prado 1984). Later studies showed the presence of metacercariae and metacestodes of the families Fellodistomidae and Phyllobothriidae in the adductor muscle, and metacestodes of Trypanorhyncha in gonads and hepatopancreas (Gomez del Prado et al. 1992). Massó (1996) concluded that there was no risk to human health.

In pectinids, some parasites cause massive infections and mortality in natural populations (Moyer et al. 1993, Whyte et al. 1994), and consequently cause significant economic losses to fisheries and mariculture by a decrease in the catch volumes and by the reduction in yields and quality as human food.

During summer 1995 (June and July), an outbreak of disease was detected in the commercial stocks of catarina scallop in B.C.S. The main sign of disease was the presence of abundant melanized spots in the adductor muscle, which resembled pepper granules (Fig 2a). Attempts to determine the aethiologic agent using standard histological techniques failed. The objective of this study was to determine the etiology of the "pimientilla" disease and ascertain if the disease represented a risk to human health.

To determine the cause of the disease, 450 specimens were submitted to the Department of Experimental Biology of the Interdisciplinary Center of Marine Sciences in July and August 1995. The specimens were collected in the two main areas of commercial exploitation of B.C.S.: Bahía Magdalena on the Pacific Coast and Bahía Concepcion on the Sea of Cortéz (Fig. 1). After collection, the specimens were kept on ice before analysis.

In the laboratory, the spots were dissected from the adductor muscle. The parasites were extracted by pressing spots between two glass slides and were fixed in AFA (70% ethanol:Formalin:Acetic acid, 80:10:10). Specimens were stored in 70% ethanol, stained with Gomori Trichromic, and mounted in synthetic resin. The description of the aethiological agent was based on nine mounted organisms. Measurements were made with an ocular micrometer.

There was a high prevalence of diseased scallops from both sites. Ninety-eight percent of specimens analyzed show the characteristic signs of disease (small brownish spots in the adductor muscle). The spots in the muscle were caused by the presence of embedded cysts. The number of cysts per adductor muscle varied between a few to more than 800. From each cyst, we recovered a metacercaria, which was identified as *Stephanostomum* sp.

The characteristics of the metacercariae (Fig. 2b) recovered were: body 764 μm (554–1291 μm) long and 280 μm (228–316 μm) width, tegumental spines, terminal oral sucker of 241 μm (208–292 μm) long and 235 μm (180–361 μm) with the mouth located in the mid-region of the body, and two rows of 28 oral spines each (56 total) 43 μm (36–56 μm) long and 18 μm (12–21 μm) width. The prepharynx was 234 μm (50–476 μm) long and the pharynx 132 μm (98–157 μm) long and 122 μm (92–146 μm) width, continuing posteriorly to the intestinal bifurcation, that is in the posterior forebody. The caecae reach close to posterior extremity and end blindly.

The ventral sucker is preequatorial with diameter of 264 μm (201–362 μm). There are a pair of dorsal ocelli between the oral sucker and the pharynx. There are reproductive organs in immature stage in the posterior third of the body, and two testes in line, the ovary is located anterior to the testes. The genital pore is just

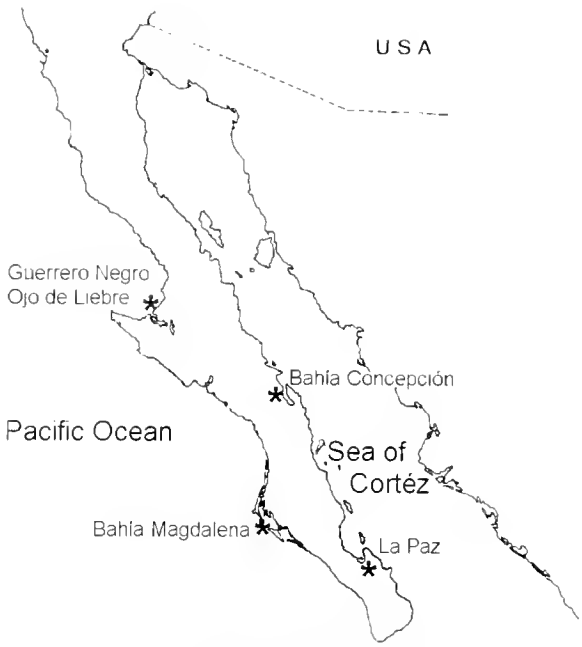

Figure 1. Catarina scallop *Argopecten ventricosus (circularis)* sampled areas.

before the body middle line and the excretory vesicle in I-like form.

The genus *Stephanostomum* includes numerous parasites of marine teleosts, generally warm-water species (Quinteiro et al. 1993). Species determination in adult stages is based on morphological characteristics, e.g., the number, shape, and position of peristomial spines, the relative size of the oral and ventral suckers, the number and distribution of vitellaria, the size of the cirrus sac and the genital atrium, the distance between gonads, the presence or absence of uroproct, and the egg size (Stunkard 1961).

In the metacercariae recovered from catarina scallop, the abundance of cephalic spines (56) is similar to that of *S. pronicropsi* (50–58), *S. baccatum* (56–60), and *S. fistulariae* (56). However, *S. pronicropsi* has been reported only from the North Atlantic and although *S. baccatum* has been reported in the Pacific Ocean of North America, the metacercariae has only been reported in sole (Table 1). *S. fistularinae* has only been reported from Japan, and the life cycle has not been described yet. The number of cephalic spines in the 13 species of *Stephanostomum* found in the Pacific Ocean of North America (Love and Moser 1983) do not coincide with the metacercariae found in the catarina scallop. The results obtained in the present study indicate that the specimens recovered from catarina scallop have unique characteristics. Although there is not sufficient information available for the complete identification at species level, we can't discard the possibility of it being a new species.

The complete cycle of *Stephanostomum* comprises three principal stages: redia, metacercaria, and adult, all of which require different hosts. Most species of the genus have been described from adult specimens, and intermediate hosts are unknown (Table 1 shows the species for which the intermediate hosts are known). The complete cycle is known for *Stephanostomum tenue* and *Stephanostomum baccatum* and the metacercaria, and adults of *Stephanostomum japonicum*, *Stephanostomum bicoronatum*, *Stephanostomum hispidum*, *Stephanostomum carangium*, and *Stephanostomum lophii* have been described as parasites of fish (Table 1). In general, the rediae of *Stephanostomum* are parasites of gastropods and the other stages are parasites of marine fish. This is the first record of a bivalve mollusk that acts as an intermediate host for *Stephanostomum*.

Catarina scallops inhabit sandy bottoms, coexisting with some gastropods, and is preyed upon by fish. To date in B.C.S., gastropods have not been reported as hosts of rediae of *Stephanostomum* nor have fish been reported as a definitive host. However, the species *S. californicum* and *S. dentatum* have been reported in La Jolla, California from the intestinal tract of the yellowfin croaker (*Umbrina roncador*) and the California halibut (*Paralictis californicus*), respectively (Manter and Van Cleave 1951). Nevertheless, the number of cephalic spines in the adults of these two species does not match the number on the metacercaria from catarina scallops.

No metacercariae were found alive during the dissection. The method employed for transporting the scallops during our analysis is similar to that used commercially, suggesting that the parasite would not survive during commercial processing and shipping. Furthermore, the life cycle of this parasite does not include mammals. Thus, we can discard concerns for human health. However, a bigger problem is the decrease in the quality of the market product. Because when the infestation is severe, the product will not meet the standards of quality required for international commercialization.

The catarina fishery is an important economic activity in Baja California Sur. The recent collapse of natural stocks has been

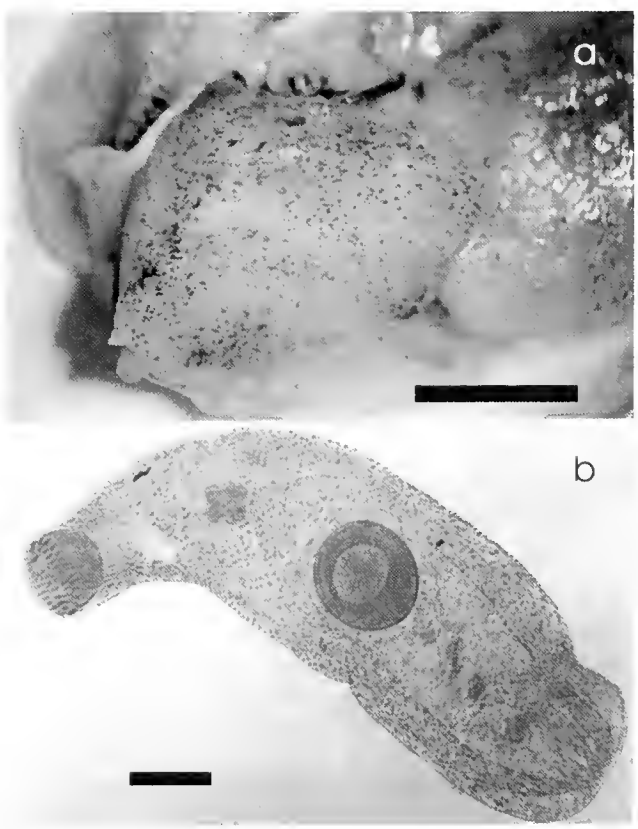

Figure 2. The "Pimientilla" disease of catarina scallop *Argopecten ventricosus (circularis)*. (a) Melanized spots on a dissected adductor muscle (bar = 1 cm). (b) *Stephanostomum* sp. metacercaria, the causal agent, stained with Gomori Trichromic (bar = 100 µm).

TABLE I.

***Stephanostomum* species for which the intermediate hosts are known.**

Species	Intermediate Host			Author
	Redia	Metacercaria	Adult	
Stephanostomum tenue	*Nassa obsoleta*	*Menidia menidia notata*	Marine fishes	Martin (1939) Caballero & Caballero (1952)
Stephanostomum baccatum	*Buccinum undatum* *Neptunea decemcostatum*	Sole	Marine fishes	Schell (1970)
Stephanostomum japonicum	Unknown	*Engraulis japonicus* *Lotella phycis*	Fish	Caballero & Caballero (1952)
Stephanostomum hispidum	Unknown	*Sebastes tompsoni*	Fish	Ohnishi et al. (1991)
Stephanostomum bicoronatum	Unknown	*Acanthogobius hasta* *Sciaena sp.* *Taenioides lacepedi*	Marine Fish	Caballero & Caballero (1952)
Stephanostomum carangium	Unknown	*Hippocampus coronatus*	Marine Fish	Caballero & Caballero (1952)
Stephanostomum lophii	Unknown	Marine fish	*Lophius piscatorius*	Quinteiro et al. (1993)
Stephanostomum sp.	Unknown	Catarina scallop	Unknown	Present report

associated with overfishing and adverse environmental conditions. Consequently, the cultivation of this species is gaining importance (Castro-Ortíz 1993). During our study, we found a high prevalence of infected scallops and a high intensity of cysts embedded in the adductor muscle. Further research is necessary to determine the effect of *Stephanostomum* outbreaks on natural and cultured populations and the risks that this parasite may impose on scallop culture.

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INFLUENCE OF THE GONADAL CYCLE AND FOOD AVAILABILITY ON POSTMORTEM CHANGES IN GLYCOGEN, ADENOSINE TRIPHOSPHATE, HYPOXANTHINE, AND THE 260/250 ABSORBANCE RATIO IN ADDUCTOR MUSCLES FROM SCALLOP *AEQUIPECTEN TEHUELCHUS* (D'ORBIGNY, 1846)

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ABSTRACT The influence of food availability and the reproductive cycle on the postmortem levels of glycogen, adenosine triphosphate (ATP), hypoxanthine, and the 260/250 absorbance ratio in extracts of adductor muscles from scallop (*Aequipecten tehuelchus*) was investigated. Phytoplankton production, measured as chlorophyll "a" concentration, was high in early autumn (March to April) and early spring (September to October). Food availability was low in winter and summer. Specimens in good and poor biological condition, according to the determination of chemical composition and energy content, were found in periods of high and low food availability, respectively. Glycogen and ATP levels were higher in muscles from specimens in better biological condition. The rate of decrease of both glycogen and ATP was not dependent on the biological condition. The values of the 260/250 absorbance ratio were higher in extracts of cold-stored adductor muscle from scallops in better biological condition. Irrespective of the biological condition hypoxanthine increased after 24 h in cold-stored muscles. Thereafter, the highest increments were observed in muscles from specimens in the best biological condition.

KEY WORDS: ATP, biological condition, glycogen, gonadal stages, hypoxanthine, *Aequipecten tehuelchus*

INTRODUCTION

A large amount of scallop is consumed as fresh material and is used as material for dried, smoked, or canned products. In addition to that use, the consumption of frozen scallop adductor muscle has been remarkably increasing in the last years. On the other hand, it is widely accepted that nucleotides influence the taste and flavor of fish and mollusks meat (Matsumoto & Yamanaka 1990).

Nucleotide degradation in postmortem fish muscle proceeds as follows: adenosine triphosphate (ATP) → adenosine diphosphate (ADP) → adenosine monophosphate (AMP) → inosine monophosphate (IMP) → inosine (HxR) → hypoxanthine (Hx) (Kassemsarn et al. 1963, Ehira & Uchiyama 1987). In marine invertebrates, a major route for conversion of AMP to HxR via adenosine (Ado) rather than IMP was proposed (Saito et al. 1958, Arai 1960, Hitlz & Dyer 1970). However, several reports are available on the accumulation of IMP in postmortem molluskan muscle (Sakamoto et al. 1973, Nakamura et al. 1976, Suwetja et al. 1989). Contrary to the results of Saito et al. (1958) and Arai (1960), Suwetja et al. (1989) detected a small amount of IMP in the same species of cephalopods and bivalves. The reason for these apparently contradictory reports could be the different biological condition (season, sex, food availability, physiological condition, etc.) of the specimens analyzed. De Vido de Mattio et al. (1992) reported that glycogen and ATP levels were higher in adductor muscles from the bivalve mollusk *Aulacomya ater ater* (Molina) in good biological condition. In this work, it was also reported that the values of the 260/250 absorbance ratio in perchloric acid muscle extracts from *Aulacomya* specimens in good biological condition were significantly higher than the values from specimens in poor biological condition. Because of that, it was suggested that the biological condition of *Aulacomya* influences the ratio among ATP and catabolite compounds of ATP breakdown in stored muscles (De Vido de Mattio et al. 1992). A poor biological condition in the bivalve mollusk *Aulacomya* was found during the consumption of energy reserves for metabolic requirements and the development of the gonads in winter in response to insufficient availability of food (De Vido de Mattio 1980). Therefore, more investigation is necessary to determine the real influence of either the reproductive cycle or the availability of food on postmortem changes in ATP and its catabolites in invertebrate adductor muscles.

The scallop *Aequipecten tehuelchus* is a rhythmic hermaphrodite with both gonadal growth and gonadal resting periods (Christiansen & Olivier 1971, Lasta & Calvo 1978). Unlike *Aulacomya*, the gonadal growth period of *Aequipecten* occurs with food availability in the environment. The purpose of the present work was to study the influence of both the gonadal cycle and availability of food on postmortem changes in ATP and catabolites compounds of ATP breakdown in adductor muscles of the scallop *Aequipecten tehuelchus* stored at 2–4°C.

MATERIALS AND METHODS

Specimens of *Aequipecten tehuelchus* (D'Orbigny, 1846) were collected in the San Jose Gulf, Chubut, Argentina. Mature specimens, 70 mm in length, were selected. Resting and gonadal growth periods were determined by macroscopic observation and histology of gonads (Lasta & Calvo 1978). After cleaning the shells, adductor muscles were dissected and carefully freed from adhering pancreatic and liver tissues. Adductor muscles were stored at 2–4°C for up to 10 days. Two experimental runs with mollusks in different biological conditions were performed. At zero time and at different periods of storage, adductor muscles from about 30 speci-

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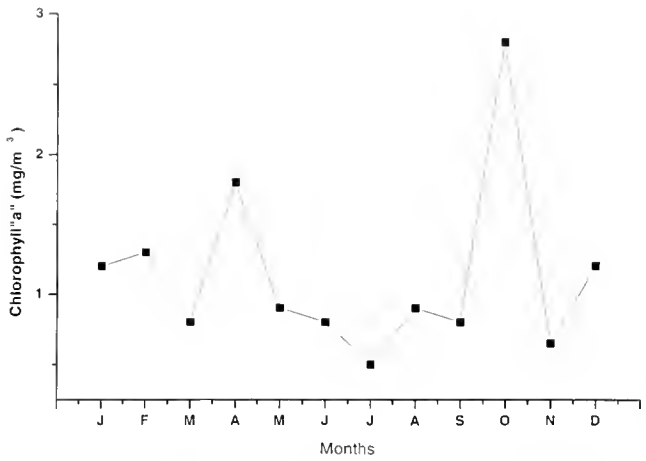

Figure 1. Seasonal changes in phytoplanktonic production expressed as chlorophyll "a" concentration. Each point is a mean of three determinations. Average relative deviations < ±3%.

mens were cut into small pieces and thoroughly mixed to ensure homogeneity. Aliquots were taken for the following studies.

Proximal Chemical Composition

Lipids were determined from fresh homogenized material using Soxhlet (Pearson 1971). Water content was measured by drying the tissues at 100°C until a constant weight was reached. Glycogen content was determined colorimetrically using the anthrone reagent, according to the method described by Fraga (1956). Protein content was measured by the Lowry method (Lowry et al. 1951). Ash content was measured by ashing the tissue in a muffle furnace at 500°C. In all cases, the results correspond to the mean of triplicate determinations. They are expressed as percentages of the wet weight.

The energy values of the muscles were determined by the percent chemical composition, with Rubner's coefficients (e.g., lipids 9.45, glucides 4.20, and proteins 5.65) (Winberg 1971).

ATP Content

A portion of pooled, cut, and mixed muscles was homogenized with 10% trichloroacetic acid (TCA). The homogenate was centrifuged at 700 × *g*. The residue was extracted again with 5% TCA. The supernatants were combined and neutralized with a 20% KOH solution under cooling with ice. The neutralized extract was centrifuged and the precipitate was washed with 5% TCA neutralized with KOH. Aliquots of supernatants were taken to measure ATP by the luciferin-luciferase assay as described by Romano and Laborde (1978), adapted for bivalve adductor muscles. A PICO-ATP luminometer (Jovin and Ivon, Ste. France) was employed for light peak measurement.

Hypoxanthine

Ten grams of muscles were homogenized in a Waring blender with 30 mL cold 7% perchloric acid for 1 min, then filtered through Whatman 1 filter paper. The extracts were neutralized with 30% KOH. The $KClO_4$ precipitate was removed by centrifugation and the volume of neutralized extracts was adjusted to 50 mL. Hypoxanthine concentration was estimated by xanthine oxidase activity according to the procedure reported by Jones et al. (1964).

The 260/250 Absorbance Ratio

The 260/250 absorbance ratios were determined as the ratio of the absorbance of perchloric acid muscle extracts at 260 nm to that at 250 nm (Korhonen et al. 1990). One gram of tissue was homogenized in a Waring Blender with 25 mL cold 20% perchloric acid for 1 min, then filtered through Whatman 1 filter paper. The filtrate was diluted with three volumes of distilled water. Absorbance was measured immediately using a Beckman DU8 spectrophotometer at wavelengths of 250 and 260 nm.

Statistical Analysis

eans were compared by analysis of variance and the Student's *t*-test. Differences between slopes were determined by the Student's *t*-test.

RESULTS AND DISCUSSION

Because bivalve mollusks are primary consumers, the phytoplankton supplied with particulate organic material constitutes the main source of food. The changes during the year in the phytoplanktonic production, expressed as chlorophyll "a" concentration, are shown in Figure 1. High phytoplankton production can be observed in early autumn (1.8 mg · m^{-3}) and early spring (2.8 mg · m^{-3}). Food availability was low in winter and summer. Lasta and Calvo (1978) reported that the reproduction of the *Aequipecten tehuelchus* occurs as follows: by the end of winter and during early spring, most of the individuals show the first stages of gonad ripening. At the beginning of summer (i.e., during December), the ovocytes increase rapidly in size and ripe animals begin to spawn naturally. The spawning season lasts for most of the summer in the southern hemisphere, from December to February. The gonadal

TABLE 1.

Biochemical composition and energy value of adductor muscles from *Aequipecten tehuelchus* at different stages of the reproductive cycle.

Reproductive cycle period	Biochemical composition (%)					Energy value (Kcal/g)
	Lipids	Glycogen	Proteins	Ash	Water content	
Gonadal growth	0.63	5.33	15.89	1.78	74.50	1.17
Gonadal resting[a]	0.36	7.31	17.70	1.91	76.25	1.34
Gonadal resting[b]	0.35	2.70	17.06	1.62	78.70	1.10

Each value is the mean of three determinations. Average relative standard deviations less than 3%.

[a] Beginning of gonadal resting period.

[b] End of gonadal resting period.

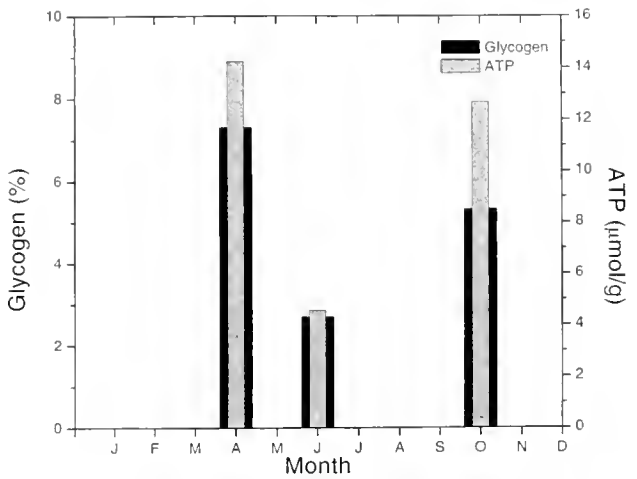

Figure 2. Seasonal changes in ATP and glycogen content in adductor muscles of scallop. Each value is a mean of six determinations. Average relative deviations < ±3%.

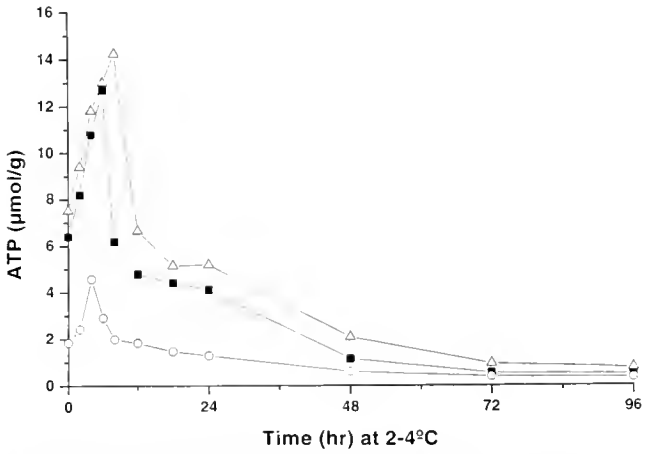

Figure 4. Changes in ATP content in cold-stored muscles. For other details see legend to Figure 3.

resting period starts in early autumn and finishes at the end of winter. Like many other bivalves, scallops have seasonal cycles of energy use that are intimately associated with the reproductive cycle (Ansell 1974, Barber & Blake 1991). The biological condition of scallop at different stages of the reproductive cycle is shown in Table 1. The highest values of the relative percentages of glycogen and proteins were observed in the adductor muscles of specimens at the beginning of the gonadal resting period. Correspondingly, the energy content was higher in these samples and therefore specimens in this period present the best biological condition. The relative percentages of the different components of the adductor muscle and the energy content, except the relative percentage of lipid, were slightly lower in specimens in the gonadal growth period than in those caught at the beginning of the gonadal resting period (Table 1). Because of that, specimens caught during the gonadal growth period remain in good biological condition. Variations in body components indicate either accumulation or use of storage substrates (Barber & Blake 1991, Martinez & Mettifogo 1998). In *Chlamys septemradiata* and *Pecten maximus* energetic reserves of the adductor muscles are used for gonadal maturation, producing an important decrease in the weight of the muscles (Ansell 1974, Comely 1974). Conversely, in *Aequipecten tehuelchus* the weight of the muscles either did not decline or declined slightly during the gonadal growth period (Lasta & Calvo 1978). The results shown in Table 1 and those of Lasta and Calvo (1978) suggest that little of the energetic reserves of the adductor muscles were used for metabolic and reproductive purposes during the period of highest gonadal activity. It could be suggested that the energy requirements for reproduction of *A. tehuelchus* are mainly satisfied by the food taken from the environment. In agreement with these results, it was reported that during artificial conditioning of the sea scallop, *Placopecten magellanicus*, the mean weight of the adductor muscle was more than twice the weight observed in the wild specimen during gametogenesis. This indicates that the scallops were accumulating reserves despite the stress of gamete production (Paon & Kenchington 1995).

Glycogen constitutes about 93% of the total glucides that are present in adductor muscles (De Vido de Mattio 1980). The major anaerobic energy source in postmortem muscle is the breakdown of glycogen. As it can be seen in Figure 2, high and low levels of both glycogen and ATP were found in adductor muscles in periods of high and low food availability, respectively. These results are additional evidence of those energetic reserves that were stored in adductor muscles in periods with high food availability in the environment. Glycolysis profiles of cold-stored adductor muscles

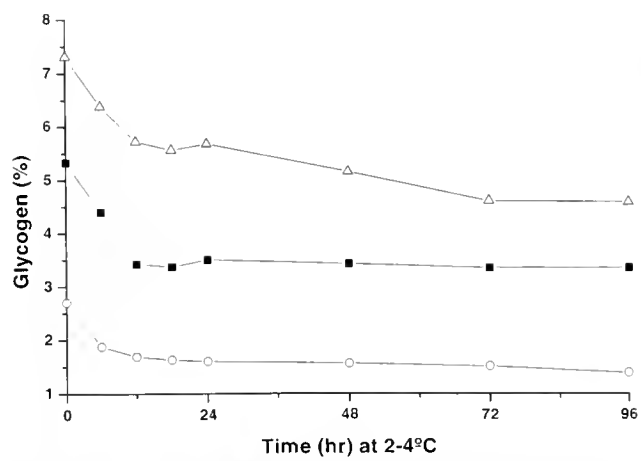

Figure 3. Changes in glycogen content in cold-stored muscles: (■) gonadal growth period, (△) beginning of gonadal resting period, (○) end of gonadal resting period. Glycogen content is g/100 g muscle. Each point is a mean of six determinations. Average relative standard deviations < ±3%.

TABLE 2.

Changes in 260/250 absorbance ratio of muscles extracts from *Aequipecten tehuelchus* at different biological conditions.

Hour at 2–4°C	Poor biological condition	Good biological condition
0	1.22 ± 0.01*	1.26 ± 0.01*
24	1.17 ± 0.01*	1.20 ± 0.01*
48	1.12 ± 0.01*	1.16 ± 0.01*
72	1.05 ± 0.01*	1.08 ± 0.01*
96	1.03 ± 0.01**	1.05 ± 0.01**
120	1.01 ± 0.01**	1.03 ± 0.01**

Result are means ± standard deviations ($n = 4$).
* $P < 0.01$.
** $P < 0.05$.

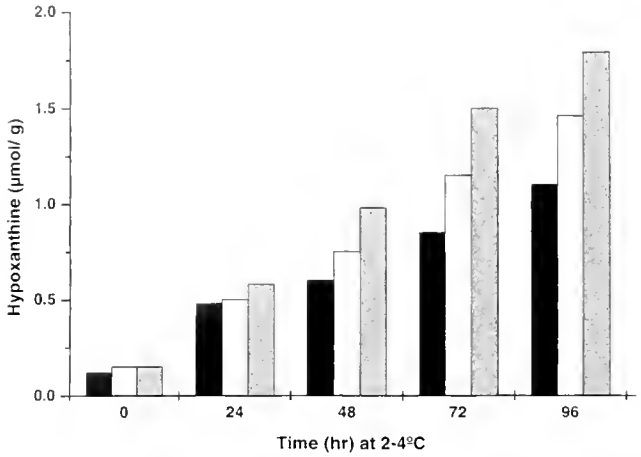

Figure 5. Changes in hypoxanthine content in cold-stored muscles: (□) gonadal growth period, (▨) beginning of gonadal resting period, (■) end of gonadal resting period. Each value is a mean of six determinations. Average relative standard deviations < ±3%.

from *A. tehuelchus* in different biological conditions are shown in Figure 3. Irrespective of the biological conditions of the specimens, glycogen content decreased within the first 12 h of storage. Glycogen content decreased slowly thereafter up to the end of storage. No significant differences ($P < 0.05$) in the slope of the curves were found within the first 12 h of storage. The results shown in Figure 3 indicate that the glycolysis rate is not dependent on the initial glycogen content of adductor muscles. Similar results were observed with *Aulacomya* adductor muscles stored at 2–4 C (De Vido de Mattio et al. 1992).

Changes in the ATP concentration in stored muscles are shown in Figure 4. The ATP content increased for a short time after death because the nucleotide is regenerated by degradation of arginine phosphate prior to destruction of ATP. As expected, the maximum ATP content in muscles was dependent on the biological condition of the specimens. After the maximum was reached, the ATP content fell sharply up to 12 h. No significant differences were found in the slopes of ATP decrease. Thereafter, nucleotide levels decreased slightly up to 72 h of storage. The ATP content was unchanged after 72 h.

Based on the measurement of the postmortem conversion of adenosine nucleotides to derivates IMP, HxR, and Hx, the R value was defined as the 250/260 absorbance ratio. Adenosine nucleotides show maximum absorption at 259–260 nm, and IMP, HxR, and Hx at 249–250 nm. The inverse of the R value, the 260/250 absorbance ratio, was successfully used to determine the effects of antemortem stress on the rate of onset of rigor mortis and associated biochemical changes in fish muscle (Korhonen et al. 1990). In addition, it was also successfully used to demonstrate the influence of the biological condition of the bivalve mollusk *Aulacomya* on the ratio among ATP and catabolite compounds of ATP breakdown in cold-stored muscles (De Vido de Mattio et al. 1992). The 260/250 absorbance ratios of perchloric acid extracts of stored molluskan adductor muscles are shown in Table 2. The values of the 260/250 absorbance ratio of about 1.26 and 1.22 were obtained at zero time of storage for samples from specimens in gonadal resting period with good and poor biological condition, respectively. These values were significantly different ($P < 0.01$). Khan and Frey (1971) reported initial values of about 1.2 for well-rested beef and pork, the values subsequently declining to 0.89 or lower upon the passing of the samples into rigor. Korhonen et al. (1990) reported initial values of about 1.07 and 0.97 for muscles from unstressed and stressed fish, respectively. The initial values found by us in adductor muscles from scallop were similar to those of beef and pork and slightly lower than those reported for adductor muscles of *Aulacomya*. During the storage, the values of the 260–250 absorbance ratio of muscle extracts from specimens in poor biological condition were significantly lower than the values of muscle extracts from specimens in good biological condition ($P < 0.01$ at 24, 48, and 72 h; $P < 0.05$ at 96 and 120 h) (Table 2). In the experiments shown in Table 2 only specimens in gonadal resting periods were used. Therefore, an influence of the food intake on postmortem changes in the ratio among ATP and catabolite compounds of ATP breakdown in stored muscles might be suggested. The profiles of Hx production in cold-stored adductor muscles of scallop are shown in Figure 5. Irrespective of either gonadal stage or biological condition of specimens, Hx levels linearly increased about 230% during the first 24 h. Thereafter, the increment in the Hx content was higher in muscles of scallop in better biological condition. In agreement with these results it was reported that the Hx levels reached since day 5 in cold-stored adductor muscles from *Aulacomya* in good biological condition were higher than the levels from specimens in poor biological condition (De Vido de Mattio et al. 1992).

The results of this paper demonstrate that glycogen and ATP levels were higher in specimens in good biological conditions. The rate of decrease of both glycogen and ATP in adductor muscles was not dependent on the biological conditions of the scallops. Food availability influences both the biological condition of scallops and the postmortem changes in the ratio among ATP and catabolite compounds of ATP breakdown in stored muscles.

ACKNOWLEDGMENTS

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KING SCALLOP (*PECTEN MAXIMUS*) DEPURATION TRIALS

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ABSTRACT Preliminary trials were undertaken to assess the potential for depuration (purification) of diver harvested king scallops (*Pecten maximus*) under a range of temperatures and shellfish to water ratios. Scallops taken from class B waters were held for 42 hours in standard design depuration systems. Bacteriological analysis indicated a significant decrease in *E. coli*. and total viable count (TVC) at all temperatures and water ratios after the 42 hour depuration period. Mortalities at temperatures above 20.5°C and increased ammonia levels at low shellfish to water ratios indicated that acceptable depuration conditions lie between 6.6°C and 15.6°C at shellfish to water ratios in excess of 1:12. Scallops held upside down also depurated effectively, although TVC indicated that in this orientation depuration was less effective than in scallops held the right way up.

KEY WORDS: bacteriological analysis, depuration, *Pecten maximus*

INTRODUCTION

In order to reduce the risks associated with eating raw or lightly cooked bivalve mollusks the food safety (hygiene) regulations 1998 (Fishery Products and Live Shellfish) require that bivalve mollusks from class B waters be depurated (purified) to reduce bacteriological contamination before releasing for human consumption. The Seafish Industry Authority (SEAFISH) has established protocols for depuration, based on temperature, salinity, flow rates, oxygen levels and loading density, for a number of shellfish species. These include; cockles (*Cerastoderma edule*), mussels (*Mytilus edulis*), oysters (*Ostrea edulis* and *Crassostrea gigas*) and razor clams (*Ensis* sp.) (Boulter et al. 1994, SEAFISH 1997). While protocols for de-gritting king scallops (*Pecten maximus*) have been set (McNamara 1996) there are currently no protocols for depuration of king scallops.

Currently there are three scallop farms in Northern Ireland that either have full culture licenses or are in the process of receiving a culture license. One of these farms is known to be in a "Class B, shellfish harvesting area" and at least one other farm is likely to be classified as "B" when production starts. It is therefore imperative that depuration criteria be set for scallops in Northern Ireland in the near future.

An initial trial aimed at depurating king scallops was run in August 2000, using SEAFISH standard design experimental depuration tanks.

Aims

- To determine whether depuration of king scallops is possible using theoretically normal conditions (salinity 31 psu and temperature 10°C–15°C)
- To establish the temperature range over which king scallops will depurate under normal saline conditions (31 psu)
- To establish the effect of shell orientation on king scallop depuration
- To determine a suitable shellfish to water ratio for depuration
- To determine the effect of depuration conditions on post depuration survival

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MATERIALS AND METHODS

On October 17, 2000 approximately 100 kg of diver harvested king scallops were collected from Mulroy Bay, Co. Donegal and transported by road to the Centre for Marine Resources and Mariculture (C-Mar), Portaferry, Northern Ireland, a journey lasting 3.5 hours.

On October 18, the scallops were transferred to plastic trays (NW trays) (dimensions 480 mm × 480 mm × 60 mm with 4 mm square holes), in a cupped side down position, at a density of 10 scallops per layer. These trays are used for scallop cultivation in the UK and Ireland (Hardy, 1991). Eight stacks of trays, seven layers deep were then transported 25 miles to Bangor, County Down by road. They were then transported by boat and placed on the sea bed at a shellfish cultivation site off Greencastle, inner Belfast Lough. (50°40.50′ N, 005°52.77′ W) This site is known to produce "Class B" mussels.

On November 28, four tray stacks of scallops were recovered from Belfast Lough. On December 11, the remaining four tray stacks were recovered. The surviving scallops were removed from the trays immediately and placed cupped valve down in fish boxes and covered with damp sacking. A sample of the surviving scallops was packed in a cool box and sent by courier to Aqualab, Killybegs, County Donegal, for microbiological analysis. The remaining scallops were returned to the C-Mar Laboratory. Emersion time for scallops used for depuration trials did not exceed 3.5 hours.

On arrival at Portaferry the scallops were weighed and placed into NW trays at a maximum of 10 scallops per tray, approximately 30 live scallops (5kg) for each trial (Table 1).

The trays were then transferred to experimental scale depuration tanks (Seafish Industry Authority standard design) for 42 hours. Temperature, salinity, ammonia ($NH3 + NH_4^+$) and oxygen levels were monitored throughout the trials. Salinity measurements were recorded with a WTW LF325 conductivity meter, ammonia measurements were made with a Bran Leubbe (Germany) autoanalyser (Salicylate method) and oxygen and temperature measurements were taken with a WTW Oxi 340 oxygen meter.

On completion of the depuration trial the tanks were drained and the scallops removed. All scallops were weighed and assessed for survival.

Samples of 10 scallops, randomly selected from each trial, were

packed in a cool box and sent by courier for post depuration microbiological analysis at Aqualab. Samples were screened in triplicate for *E. coli* (MPN $100g^{-1}$ flesh), and Total Viable Count (ACC cfu g^{-1})(30°C 48 h). Pre-depuration samples were also screened for Salmonella.

The remaining scallops were returned to NW trays in a flow through system with ambient seawater (8.5°C and 31 psu) and held for a further seven days to assess survival post depuration.

The trial aimed to assess three variables within the depuration system:

1. Temperature
   temperatures of 5, 6, 10, 14, 16, 20 and 24°C were tested at 31 psu and a shellfish to water ratio of 1:15;
2. Shellfish to Water ratio
   calculated as 1kg of animals in 1l of water being equivalent to a ratio of 1:1. Ratio's tested were 1:3, 1:6, 1:9, 1:12 and 1:15. Temperatures were held between 13°C and 14°C;
3. Shell orientation
   scallops were held either cupped valve down (right way up) or cupped valve up (upside down) at 31 psu and 14°C and shellfish to water ratio of 1:12;

Flow rates within the systems were held at a constant 750 l hr^{-1}.

RESULTS

Pre Depuration

Pre depuration samples (Table 1) indicated that on both sample dates scallops collected from Belfast Lough had *E. coli* counts that would classify them as "Class B". However, scallops collected on November 28 had significantly lower *E. coli* levels and TVC (1910 MPN $100g^{-1}$ and 4.0×10^3 cfu g^{-1} respectively) than those collected December 11, (2733 MPN $100g^{-1}$ and 499.4×10^3 cfu g^{-1} respectively). No salmonella was found.

Temperature

The investigation showed depuration at temperatures between 6.6°C and 24.5°C reduced *E. coli* levels in scallops from 1910–2733 *E. coli* $100g^{-1}$ of flesh (Class B) to a level of around 30 *E. coli* $100g^{-1}$ (Class A) in 42 hours (Table 1). Depuration at 4.5°C reduced *E. coli* levels to 437 *E. coli* $100g^{-1}$, significantly less than at higher temperatures and not sufficiently for the animals to reach the *E. coli* level required for A classification.

TABLE 1.

Pre and post depuration TVC ($\times 10^3$ g^{-1}) and *E. coli* (100 g^{-1}) counts for scallops (*Pecten maximus*) depurated at temperatures between 5°C and 20°C upside down and in shellfish to water ratios between 1:3 and 1:15 (14°C).

Trial	Pre dep. TVC ($\times 10^3$ g^{-1})	Pre dep. E. coli (100 g^{-1})	Post dep. TVC ($\times 10^3$ g^{-1})	Post dep. E. coli (100 g^{-1})
Temp. 5	494.3	2733	2.8 ± 1.3	437
Temp. 6	4.0	1910	0.6 ± 0.1	32
Temp. 10	4.0	1910	0.8 ± 0.5	<30
Temp. 14	4.0	1910	1.0 ± 0.4	<30
Temp. 15	4.0	1910	1.1 ± 0.6	<30
Temp. 20	4.0	1910	2.3 ± 0.7	32
Temp. 24	494.3	2733	3.9 ± 3.8	<30
Upside down	494.3	2733	1.5 ± 0.4	<30
Ratio 1:3	494.3	2733	1.5 ± 0.4	<30
Ratio 1:6	494.3	2733	2.6 ± 0.5	<30
Ratio 1:9	494.3	2733	2.7 ± 1.5	<30
Ratio 1:12	494.3	2733	0.7 ± 0.2	34
Ratio 1:15	4.0	1910	1.0 ± 0.4	<30

Depuration reduced TVC at all temperatures (Fig. 1). A significant correlation ($p < 0.05$) was found between temperature and post depuration TVC for scallops depurated between 6.6 and 20.5°C.

Scallop survival was high at all temperatures (Table 2) but two mortalities and one gaping animal were observed in scallops held at 24.5°C. No mortalities were observed among the surviving scallops seven days post depuration.

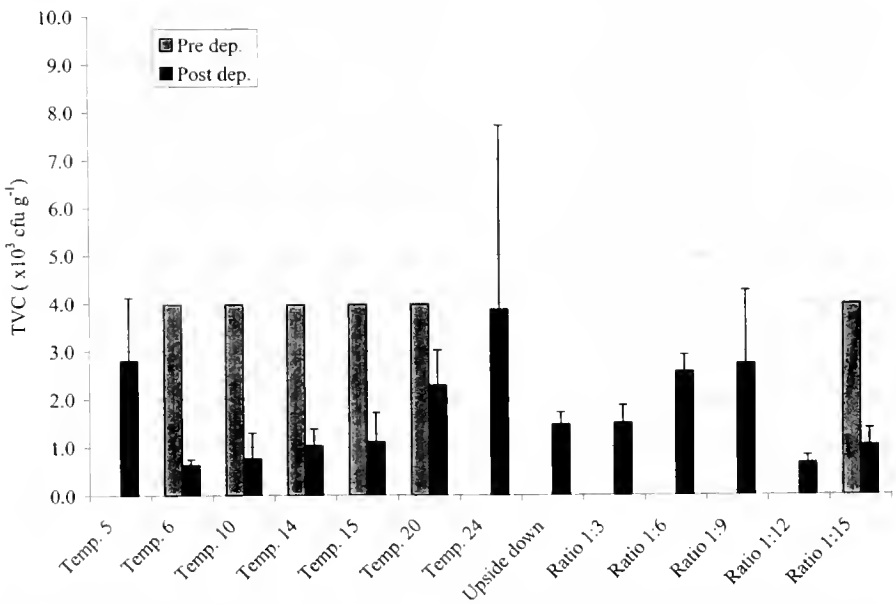

Figure 1. Pre and post depuration Total Viable Count (TVC) for scallops (*Pecten maximus*) depurated at temperatures between 5°C and 24°C, upside down and in shellfish to water ratios between 1:3 and 1:15.

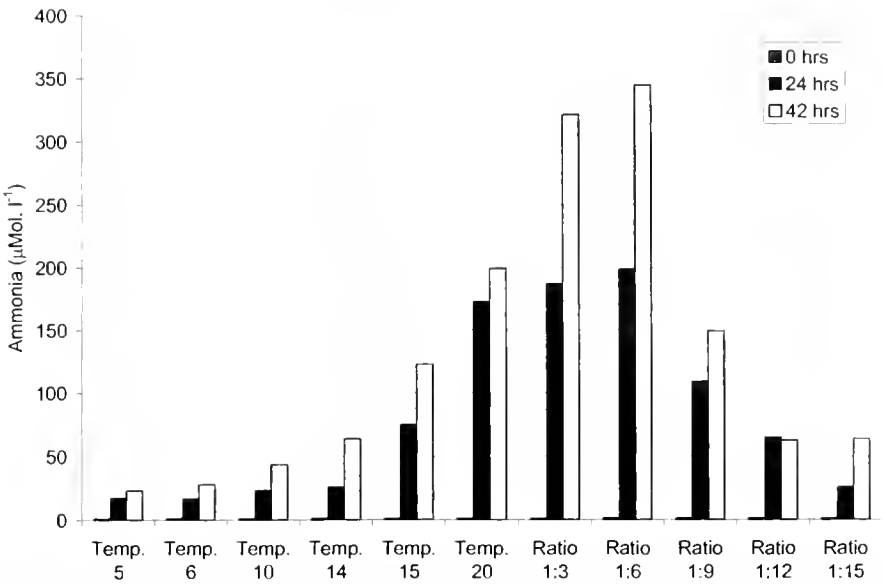

Figure 2. Pre depuration, 24 hours and 42 hours ammonia values for scallops (*Pecten maximus*) depurated at temperatures between 5°C and 20°C, upside down and in shellfish to water ratios between 1:3 and 1:15.

Oxygen levels remained in excess of 91% saturation in all tanks during the trial. Ammonia values for the trial at 24°C were contaminated and were therefore rejected. Values for temperatures between 4.5°C and 20°C increased in all tanks during the trial, showing a significant linear correlation ($r = 0.928$) between temperature and ammonia levels.

Orientation

Depuration of scallops held upside down at an average temperature of 14.2°C showed a reduction in *E.coli* from 2733 *E coli* $100g^{-1}$ (Class B) to a level of <30 *E coli* $100g^{-1}$ (Class A) in 42 hours.

Depuration also reduced TVC, but levels remained significantly higher than for scallops held at the same temperature and shellfish to water ratio the right way up ($p < 0.05$).

Oxygen levels remained in excess of 93% throughout the trial and no mortalities were observed during the depuration period or seven days post depuration.

Shellfish to Water Ratio

Depuration of scallops in shellfish to water ratios of between 1:3 and 1:15 reduced *E. coli* levels from 2733 *E coli* per 100g of flesh (Class B) to a level of around 30 *E coli* per 100g of flesh (Class A) in 42 hours.

TVC's were reduced by depuration for all shellfish to water ratios. Further analysis of the data (ANOVA and Tukey's b post hoc test) (Sokal and Rolf 1995) indicate that, for trials which started with the same pre depuration TVC, the shellfish to water ratio of 1:12 resulted in a significantly lower TVC than for the lower shellfish to water ratios ($p < 0.04$) (Fig. 1).

A single mortality was observed in each of the 1:3 and 1:6 water ratio trials during depuration. No mortalities were observed among the surviving scallops seven days post depuration.

Oxygen levels remained in excess of 91% saturation in all tanks during the trial. Ammonia values increased in all tanks during the experiment (Fig. 2) with final values in shellfish to water ratios of 1:3 (321 μmol l^{-1}) and 1:6 (344 μmol l^{-1}) approximately double those for the 1:9 ratio (149 μmol l^{-1}) and five times those for ratios of 1:12 (63 μmol l^{-1}) and 1:15 (62 μmol l^{-1}).

DISCUSSION

The study shows that it is possible to hold and depurate king scallops over a 42-hour period. However, in order to provide meaningful information to industry and to ensure that in scallops depurated for human consumption, bacterial loads are always reduced to acceptable levels, it is necessary to define more precisely that range of conditions under which scallops depurate most effectively.

TABLE 2.

Depuration temperature, number and biomass of scallops used and percentage survival, during depuration and seven days post depuration (+7) for scallops (*Pecten maximus*) depurated at temperatures between 5°C and 20°C, upside down and in shellfish to water ratios between 1:3 and 1:15 (14 C).

Trial	Temperature (°C)	No. scallops	Mass (kg)	Survival (%)	Survival +7 (%)
Temp. 5	4.6 ± 0.6	15	2.94	100	100
Temp. 6	6.6 ± 1.3	30	6.12	100	100
Temp. 10	9.9 ± 1.3	30	5.13	100	100
Temp. 14	14.1 ± 1.6	30	5.54	100	100
Temp. 15	15.6 ± 0.6	30	5.54	100	100
Temp. 20	20.5 ± 0.5	30	5.7	100	100
Temp. 24	24.5 ± 0.7	15	2.14	80	100
Upside down	14.3 ± 1.0	15	2.01	100	100
Ratio 1:3	12.9 ± 1.0	30	5.54	93	100
Ratio 1:6	13.3 ± 0.7	30	5.83	93	100
Ratio 1:9	14.2 ± 0.8	30	5.71	100	100
Ratio 1:12	13.4 ± 0.6	30	5.72	100	100
Ratio 1:15	14.1 ± 1.6	30	5.54	100	100

TABLE 3.

Maximum and minimum temperatures recommended for shellfish depuration (after SEAFISH 1997)

Species	Minimum temperature	Maximum temperature
Mussels (*Mytilus edulis*)	5°C	15°C
Native oysters (*Ostrea edulis*)	5°C	15°C
Pacific oysters (*Crassostrea gigas*)	8°C	18°C
Cockles (*Cerastoderma edule*)	7°C	16°C

The data indicate that the temperature range for scallop depuration, which ensures an acceptable reduction in bacterial levels (*E. coli.*) and does not lead to mortalities during the depuration process, is between 6.6 ± 1.6°C and 20.5 ± 0.5°C. This is greater than the range (10–18°C) recommended by McNamara (1996) for degritting scallops. Temperature ranges for depuration of other bivalves vary with species (Table 3), but can be as low as 5°C for species native to UK waters (SEAFISH 1997).

Duncan (1993) indicated that mortalities related to exposure to ammonia occurred in king scallops at levels around 518 μmol l^{-1} but post exposure mortalities were observed for constant exposure to ammonia concentrations as low as 64 μmol l^{-1} (96 hours). As exposure is not at a constant level in a depuration system, but builds up over the depuration cycle higher final concentrations may be acceptable. To ensure that scallops are not exposed, at any point during the cycle, to ammonia concentrations in excess of 64 μmol l^{-1} then a shellfish to water ratio in excess of 1:12 is recommended at a maximum temperature of 15.6 ± 0.6°C. High ammonia levels may have been the cause of the mortalities observed in shellfish to water ratios of 1:3 and 1:6.

Survival of all scallops held for seven days post depuration indicates that the depuration process does not cause stress to the animals which leads to post depuration mortality. This is important for the scallop processing industry where supply of live product to market can attract a premium.

Further work is required to assess the effects of salinity, oxygen levels and pre depuration handling on depuration performance in the king scallop.

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THE EFFICIENCY AND SELECTIVITY OF SPRING-TOOTHED SCALLOP DREDGES: A COMPARISON OF DIRECT AND INDIRECT METHODS OF ASSESSMENT

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ABSTRACT The efficiency and selectivity of spring-toothed scallop dredges was assessed using a concurrent depletion experiment and diver survey of dredge tracks on a north Irish Sea fishing ground. Two size classes of the scallop, *Pecten maximus* (L. 1758), were examined: below minimum legal landing size (MLLS), (90–109 mm shell length (SL)) and above MLLS (>109 mm SL). Estimates of efficiency from the depletion experiment (24.3% and 29.5% respectively) were consistently lower than those from the diver surveys (38.0% and 40.7% respectively). This difference appeared to be due to inherent variation in the efficiency of scallop dredges rather than bias from either technique. This emphasizes the need for error terms to be built into estimates of dredge efficiency. The diver survey also found that dredges were highly selective toward scallops greater than 90 mm SL, catching only 3.0% or less of individuals below this size. Consequently, the diver survey provided a much more accurate assessment of scallop size and age composition than dredge surveys. Dredge efficiency was also assessed for four species of benthic fauna commonly taken as by-catch in the local fishery. Estimates of efficiency from the depletion experiment were found to include a considerable amount of indirect fishing mortality. When efficiency was defined as total mortality due to fishing (the combination of catch and indirect fishing mortality), estimates from the depletion experiment and diver surveys were in close agreement. For two species, *Luidia ciliaris* (Phillipi 1837) and *Cancer pagurus* (L. 1758), these efficiency or total mortality estimates were approximately 45% and 68% respectively, emphasizing the impact scallop dredging might have on non-target species. In summary, we recommend that if possible, depletion experiments should be combined with diver surveys when assessing scallop dredge performance. Diver surveys provided additional information on dredge selectivity along with an improved measure of the variance in dredge efficiency estimates.

KEY WORDS: scallops, dredge efficiency, gear selectivity, *Pecten maximus*, depletion experiment, diver survey, stock assessment

INTRODUCTION

Accurate assessment of the state of fish and shellfish populations requires conversion of the results from stock surveys into estimates of "true" abundance (Dare & Palmer 1994). Scallop (Pectinidae) populations are often surveyed by towing commercial scallop dredges along the seabed. This technique enables relatively rapid coverage of large areas, increasing the relevance of results to commercial fisheries. In general, however, scallop dredges are relatively inefficient (estimates range from 2.1% (Caddy 1968) to 56% (Currie & Parry 1999)) and tend to be selective toward large individuals (Chapman et al. 1977, Dare et al. 1993, Fifas & Berthou 1999). The selective nature of dredges provides little information to fisheries managers on the abundance of upcoming year classes of young/small scallops.

Many factors are known to affect the efficiency and selectivity of scallop dredges. These include mechanical aspects such as dredge set up and design (e.g. teeth length, angle and spacing, belly ring diameter and teeth bar tension), operational factors such as towing speed, duration and warp length, and environmental conditions such as sea state and the substrate type of the seabed (for review see Dare et al. 1993). In addition, efficiency and selectivity may vary between scallop species according to their swimming behavior and ability to recess into the sediment (Caddy 1968, Chapman et al. 1977, Dare et al. 1993). Hence any estimate of dredge efficiency will only be relevant to the conditions under which it was measured or must be seen as an estimate of the average situation (Somerton et al. 1999).

The efficiency and selectivity of scallop dredges may be measured by either indirect or direct methods. One indirect method is to measure scallop density and population structure at a study site using a highly efficient technique such as diver surveys and then compare this to a survey of the same area using scallop dredges (e.g. Fifas 1991). Another, more commonly used indirect method is through depletion (DeLury 1947, Joll & Penn 1990, Hilborn & Walters 1992, Currie & Parry 1999). Depletion experiments are based on fitting a model to the relationship between catch rate and cumulative catch when a given area of seabed is fished repeatedly. The main advantage of depletion experiments is that they are possible on a large scale, are not limited to shallow water and are therefore particularly relevant to stock surveys and commercial fisheries. However, large numbers of replicate tows may be needed to sufficiently deplete populations and take account of variations in gear efficiency and patchiness of the stock. Regression equations used to calculate efficiency in depletion experiments can therefore be highly susceptible to outliers. Depletion experiments also provide only limited information on selectivity, as size-classes that are rarely caught will not be depleted sufficiently for efficiency to be calculated.

Direct methods for calculating scallop dredge efficiency and selectivity include monitoring the catch rate of tagged scallops seeded on the seabed at a known density (e.g. Dare et al. 1993). Possibly the most direct technique, though, is to survey dredge tracks on the seabed and compare what has been left behind with what has been caught (e.g. Caddy 1968, Chapman et al. 1977, Mason et al. 1979). Ideally, such surveys are done by divers who can count the scallops left in the dredge tracks and collect them for later measurement and ageing. Diver surveys are generally thought to be close to 100% efficient for surveying scallop populations (Mason et al. 1979, Coleman 1998), although very small individuals may be missed, which could lead to overestimates of dredge efficiency for this size class. The spatial patchiness of scallop populations (Brand 1991) may also necessitate that divers cover large areas in order to reduce variability in efficiency estimates. This generally restricts diver surveys of dredge tracks to relatively

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shallow fishing grounds and demands a high number of diver hours.

All of these methods for estimating scallop dredge efficiency and selectivity have certain advantages and disadvantages. To date, however, there have been no direct comparisons of the effectiveness of these different methods when applied to the same local scallop population at the same time. In this study we aimed to compare the outcomes and effectiveness of a concurrent depletion experiment and diver survey of dredge tracks on the same fishing ground. The work was done in the north Irish Sea off the Isle of Man, which has supported a commercial fishery for the great scallop, *Pecten maximus*, for over 60 years. Over 20 years of data from detailed stock surveys of *P. maximus* around the Isle of Man are available and interpretation of these data would be greatly enhanced by accurate estimates of dredge efficiency and selectivity. In addition to examining results for *P. maximus*, we also compared efficiency estimates and non-target mortality for four species of benthic megafauna that are commonly taken as by-catch in the Isle of Man scallop fishery. Accurate efficiency estimates for these species would allow further predictions to be made regarding the environmental impact of scallop dredging around the Isle of Man, a subject that has received considerable recent attention (e.g. Hill et al. 1999, Bradshaw et al. 2000, Kaiser et al. 2000, Veale et al. 2000b).

MATERIALS AND METHODS

Dredging for both the depletion experiment and diver surveys was done by the RV Roagan, a 24-m converted beam trawler, on the Bradda Inshore fishing ground off the Isle of Man on five days between July 12 and July 21, 2000. On each day of the study two tows were done for the dive surveys (one in the morning and one in late afternoon), with three tows in between for the depletion experiment. Weather conditions were very calm on survey days, with wind speeds less than 10 knots. Sea temperature was approximately 15°C and underwater visibility 3 to 5 m. Water depth in the study area ranged from 29m to 35m and sediment was generally a gravelly mixture of sand, shell, and mud with occasional large stones (Jenkins et al. 2001). These features are typical of many of the scallop fishing grounds around the Isle of Man. A gang of four Newhaven spring-toothed scallop dredges (total width 3.50 m) was towed off each side of the boat. Mean tooth length on the dredges at the start of the study was 76 mm (± 1 mm SE). To simulate our stock surveys and local commercial practice, all tows were approximately two nautical miles (3,704 m) in length, which at an average speed of 2.77 knots (5.13 km/hour) lasted 45 to 50 minutes. At the end of each tow the catch was landed and sorted on the deck. All scallops (*Pecten maximus*) were counted, measured, and aged to the nearest year using clearly visible validated annual shell rings (Allison et al. 1994). Numbers of the four other most common species of benthic fauna in the catch, the starfish *Asterias rubens* (L. 1758), *Luidia ciliaris*, *Porania pulvillus* (Müller 1776), and the edible crab, *Cancer pagurus*, were also counted.

Depletion Experiment

An area of seabed, two nautical miles (3,704 m) long by 40 m wide (54°07′N, 4°48′W), was selected and identified for the depletion experiment using an onboard differential global positioning system (DGPS) linked with Microplot software (Sea Information Systems, Aberdeen). Fourteen tows were done haphazardly within this area, after which time the seabed had been dredged an average of 2.45 times. Efficiency of the dredges was calculated for two size classes of scallops, 90 to 109mm shell length (SL), (under the minimum legal landing size (MLLS)) and 110mm SL or greater (above the MLLS), using the Leslie-Delury method (DeLury 1947, Hilborn & Walters 1992). This method plots the number of scallops caught during each sequential tow against the cumulative catch. The slope of the regression fitted to this plot gives a measure of the rate at which catch rates have changed during the experiment. When this slope is multiplied by the ratio of the total area of the experimental plot to the area covered by each tow, a measure of efficiency is given. In this experiment the ratio was 148,160 m^2 (the area of the plot) divided by 25,928 m^2 (the width of 8 dredges (7 m) × the length of the tow (3,704 m)), which equals 5.71428. Standard errors of these efficiency estimates were calculated from the standard error of the slope of each regression. Regression slopes for the two size classes of scallops (90 to 109 mm and >110 mm) were compared by ANCOVA with the fixed factor being size class, the dependent variable number of scallops caught per tow and the covariate cumulative catch. Differences in efficiency are indicated by a significant interaction between cumulative catch and size class.

Using the same method the overall efficiency of the dredges for catching the four by-catch species, *A. rubens*, *L. ciliaris*, *P. pulvillus*, and *C. pagurus*, was also calculated.

Diver Surveys

Diver surveys of dredge tracks were done on 10 tows of scallop dredges, conducted within an area of 2.74 km^2, approximately 200 m inshore from the depletion experiment plot. This spatial separation was necessary to ensure the safety of the divers, as the diver survey was conducted at the same time as the depletion experiment. Dredge tows were done using the same configuration and procedures described above and were of as similar distance and duration as possible. Sorting and measuring of the catch was also done as above.

During these tows the dive support vessel, RV Sula, dropped buoyed shot lines at two points, approximately 500 m apart, between the two sets of dredge tracks. This was done on both the first and second half of tows during the course of the study. After a period of 15 to 30 minutes, between two and four pairs of divers descended to the dredge tracks. The divers then swam along the tracks as far as possible given depth and air restrictions and counted all benthic fauna encountered. All scallops were also collected to allow measurement and ageing on the surface. The distance covered by the divers was calculated from DGPS readings taken from the buoyed shot lines and surface marker buoys released by the divers at the end of their surveys.

From these dive surveys the efficiency of the scallop dredges for catching scallops and the four by-catch species could be calculated using the following formula:

$$\text{Efficiency} = (\text{Density from dredges} / \text{Density from dredges + dive surveys}) \times 100$$

For the purposes of the study this formula assumes the dive surveys to be 100% efficient, which may or may not always be the case (see discussion). Data from the dive surveys were pooled for each tow (ie. from two to four pairs of divers) to allow tows to be used as replicates. The effect of number of tows (or replicates) on the magnitude and variability of efficiency estimates for catching all scallops was plotted. Along with overall efficiency estimates,

TABLE 1.

Comparison of mean scallop dredge efficiency estimates from the depletion experiment (n = 14) and diver surveys of dredge tracks (n = 10 for *Pecten maximus*).

Species	Depletion experiment				Diver surveys	
	R^2	P	Efficiency	SE	Efficiency	SE
Pecten maximus (all)	—	—	—	—	22.69	4.65
P. maximus (<90 mm)	—	—	—	—	3.01	0.66
P. maximus (90–109 mm)	0.64	0.001	24.34	5.14	37.99	3.73
P. maximus (110 + mm)	0.65	<0.001	29.54	6.29	40.07	5.05
Cancer pagurus	0.50	0.005	68.45	20.00	24.97	—
Luidia ciliaris	0.63	0.001	47.49	10.86	20.36	—
Porania pulvillus	0.18	0.013	21.37	13.14	16.27	—
Asterias rubens	<0.01	0.816	—	—	9.85	—

efficiency was calculated for three size classes of scallops; less than 90 mm SL, 90-109 mm SL and 110mm SL or greater. A 1-way ANOVA, followed by a Tukey's test, was run on these data to determine if there were any differences in efficiency for the different size classes. These efficiency estimates were also compared with those from the depletion experiment.

Size and age compositions of scallops collected during the diver surveys were also compared with those from the dredge catch to further investigate the selectivity of the scallop dredges. By combining data from the dive surveys and dredge catches, estimates of age and size composition, and density, at the study site were also calculated.

RESULTS

Depletion Experiment

The slopes of regressions between sequential catches and cumulative catch for the two size classes of scallops and for *Luidia ciliaris, Porania pulvillus* and *Cancer pagurus* were significant (Table 1). This allowed efficiency estimates to be calculated for these five groups but not for the starfish *Asterias rubens*. Regression slopes, and therefore efficiency estimates (24.3% and 29.5%), were not significantly different for the two size classes of scallops (Table 2). For the by-catch species, efficiency ranged from 21.4% for *P. pulvillus* to 68.5% for *C. pagurus* (Table 1).

Diver Survey

During the diver survey a total of 28 dives (one pair of divers per dive), covering a distance of 2.99 km, were done on the dredge tracks. From these surveys the overall efficiency of the dredges at catching scallops was calculated as 22.7% (± 4.7 SE). The magnitude and variability of efficiency estimates was relatively stable after five tows (Fig. 1). Efficiency was significantly lower for scallops less than 90 mm SL (3.0%) than for larger scallops (38.0% and 40.7%; Tables 1 and 3, Fig. 2).

TABLE 2.

Results of ANCOVA comparing regressions from the depletion experiment for two different size classes of scallop, *Pecten maximus* (90–109 mm and 110+ mm shell length).

Source	SS	df	MS	F	*P*-value
Model	60732.87	3	20244.29	65.74	<0.001
Intercept	132976.18	1	132976.18	431.81	<0.001
Size × Cum. catch	81.29	1	81.29	0.26	0.612
Size	14483.51	1	14483.51	47.03	<0.001
Cum. catch	8763.46	1	8763.46	28.46	<0.001
Error	7390.84	24	307.95		
R^2 = 0.89					

The interaction between size and cum. catch (cumulative catch) tests for differences in efficiency.

Comparison of the Depletion Experiment and Diver Surveys

For scallops greater than 90 mm SL the diver survey estimates of efficiency were approximately 10 to 15% higher than those from the depletion experiment (38.0% and 40.7% compared to 24.3% and 29.5%; Table 1, Fig. 2). For two of the by-catch species, *L. ciliaris* and *C. pagurus*, efficiency estimates from the diver surveys were less than half those from the depletion experiment (*L. ciliaris*: 20.4% compared to 47.5% and *C. pagurus*: 25.0% compared to 68.4%; Table 1). For the starfish *P. pulvillus*, however, estimates of efficiency from the two methods were similar (Diver surveys: 16.3%, Depletion experiment 21.4%). Unfortunately, due to low numbers of these by-catch species on individual dives, this dive survey data had to be pooled and results from the two methods could not be compared statistically. The diver surveys also gave an efficiency estimate of 9.3% for the starfish *A. rubens* but there was no comparable estimate from the depletion experiment.

Analysis of scallop size and age structures showed considerable differences between the diver surveys and the dredge catches.

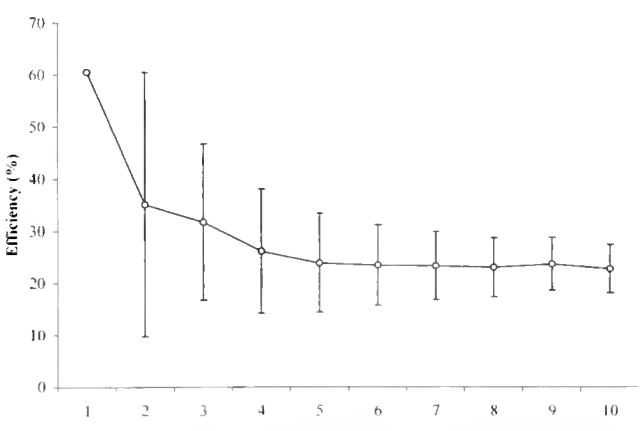

Figure 1. The relationship between the number of tows surveyed during the diver survey and estimates of dredge efficiency (mean ± SE) for all scallops, *Pecten maximus*.

TABLE 3.

Results of ANOVA comparing efficiency estimates from the diver surveys for three different size classes of scallop, *Pecten maximus* (<90 mm, 90–109 mm, 110+ mm shell length).

Source	SS	df	MS	F	P
Model	1.73	2	0.87	55.51	<0.001
Intercept	7.63	1	7.63	489.01	<0.001
Size	1.73	2	0.87	55.51	<0.001
Error	0.42	27	0.02		
$R^2 = 0.80$					

Data were Varcsine transformed to meet assumptions of normality and homogeneity of variance.
Results of Tukey's HSD test comparing efficiency estimates from the diver surveys for the three different size classes of scallop, *P. maximus*: (<90 mm) < (90–109 mm) = (110+ mm).

Scallops collected by the dredges were mainly in the 100–109 mm SL size class, while the most common size class collected by divers was 80–89 mm SL (Fig. 3a). In addition, approximately 30% of the scallops collected by divers were less than 80 mm SL, whereas these scallops were almost entirely absent from the dredge catches. This pattern was reflected by data on age composition (Fig. 3b). Most scallops caught by the dredges were 3-year-olds, while most collected by the divers were 2-year-olds. One-year-olds were not particularly common in the diver surveys (7.7%) but they were almost completely absent from the dredge catches. In both cases the combined size and age compositions were much better represented by the diver surveys than by the dredge catches. By combining the dredge catches with the diver surveys, the density of scallops, *P. maximus*, at the study site was estimated to be 4.4 per $100m^2$ (± 0.7 SE).

DISCUSSION

For the scallop, *Pecten maximus*, both the depletion experiment and the diver surveys produced relatively precise estimates of dredge efficiency that were within the range reported in other studies (Chapman et al. 1977, McLoughlin et al. 1991, Dare et al. 1993, Currie & Parry 1999). Estimates from the diver surveys, however, were moderately, but consistently, higher than those from the depletion experiment. This could be due to biases in either method, or a combination of both.

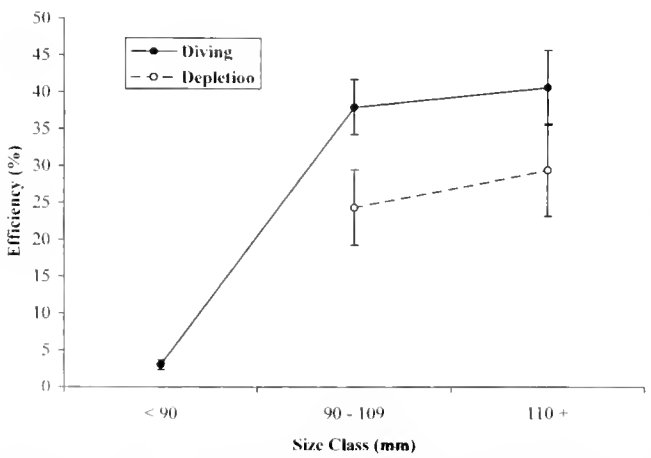

Figure 2. A comparison between dredge efficiency estimates (mean ± SE) from the depletion experiment (n = 14) and diver survey (n = 10) for three size classes of scallop, *Pecten maximus*.

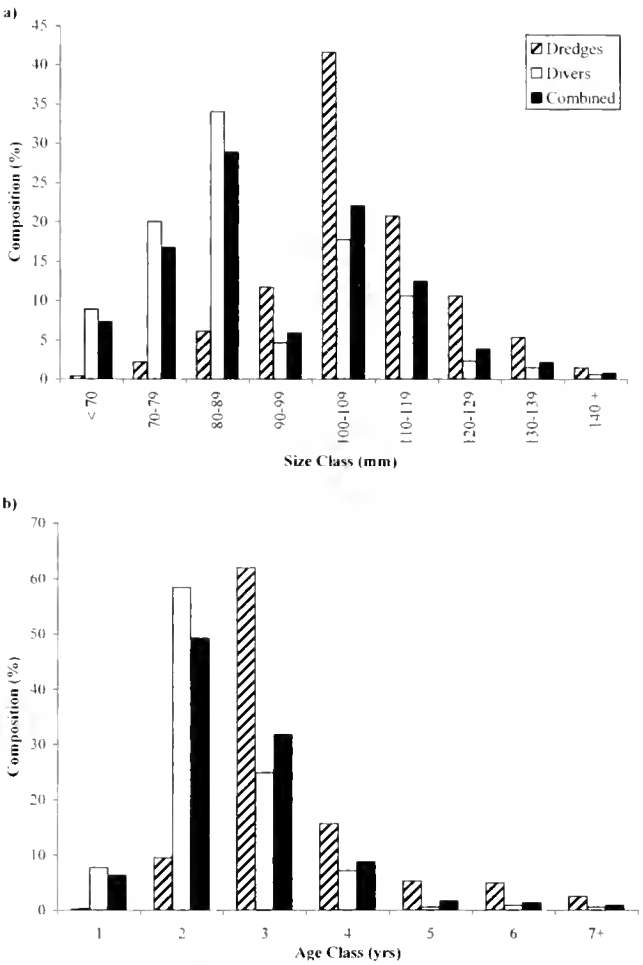

Figure 3. A comparison of the percentage composition of a) size classes and b) age classes of scallops, *Pecten maximus* collected by divers and dredges during the diver survey (n = 350 for divers and n = 2052 for dredges).

Depletion experiments are most effective when examining closed populations with little or no recruitment or natural mortality (Hilborn & Walters 1992). Although such populations rarely exist, conducting a depletion experiment over a short period of time (as in this study) will often negate the effects of recruitment and mortality (Joll & Penn 1990). Movement of the target species into or out of the study area may be more of a problem (Hilborn & Walters 1992). Given the relatively narrow width (40 m) of the depletion experiment plot, it is possible that scallops were moving into the plot during the course of the study. This would have lowered efficiency estimates. A wider plot would have reduced the potential for this to be a problem. In this study, however, we were aiming to replicate our stock surveys, which consist of long parallel tows. Without considerably more effort a wider plot would have necessitated shorter tows with frequent hauling of the gear. This may well have affected dredge efficiency estimates (Dare et al. 1993), making them less relevant to the commercial fishery. In addition, *P. maximus* is one of the most sedentary species of scallop (Brand 1991) and in a tagging study by Howell & Fraser

(1984), 60% of individuals moved less than 30 m after 18 months. Immigration is therefore likely to have had only had a small effect on our results.

Another aspect that may have affected the results from the depletion experiment is a change in the catchability of scallops during the study (Hilborn & Walters 1992). Repeated dredging of an area may cause scallops to become unrecessed, even if they are not caught (Currie & Parry 1999). This would actually have increased the efficiency of the dredges, however, whereas the depletion experiment produced lower efficiency estimates than the diver survey.

One factor that may have biased the diver survey estimates is patchiness of the scallop population (Brand 1991). Despite surveying almost 3 km of dredge tracks, 74 km of tracks were left unsurveyed. If, by chance, dive surveys focused on low-density patches of scallops, this would have elevated efficiency estimates. However, mean efficiency estimates in the dive survey were relatively stable after five tows, indicating that our level of replication and area coverage was adequate. Another possibility is that the diver survey was less than 100% efficient, which also would have elevated dredge efficiency estimates. Dive surveys are generally considered to be the most efficient method for assessing scallop populations (Mason et al. 1979, Coleman 1998), so it is not really possible to test their efficiency. In this study each diver carefully surveyed transects only 1.75m wide in underwater visibility of at least 3m. We are therefore confident that the dive survey was very close to 100% efficient, at least for scallops greater than 90mm SL. It is possible that we underestimated the number of scallops less than 90mm SL (see below), but this would only have changed our efficiency estimate of 3% for this size class to a lower value and would not have altered the conclusions of the study. Finally, differences between the two methods could have been due to variation in substrate type between the two study sites, although given their similarity and close proximity this is unlikely.

The efficiency of scallop dredges is inherently variable (Dare et al. 1993). Therefore, for the purpose of stock assessment a mean of the efficiency estimates from the two methods is probably most appropriate. The differences between them (approximately 10%) could be built in as an error term when calculating population densities. Again it must also be emphasized that these efficiency estimates are only really applicable to fishing grounds of a substrate type similar to that examined in this study. Some of the fishing grounds around the Isle of Man are much more sandy than the study site, while others are stonier. Ideally, efficiency experiments will be repeated on those other grounds in the near future.

In common with many other studies of dredge selectivity (Chapman et al. 1977, McLoughlin et al. 1991, Dare et al. 1993), we found spring-toothed scallop dredges to be very selective toward larger scallops. Dredges almost completely missed scallops below 90 mm SL, although in contrast to some other studies (e.g. Mason et al. 1979, Dare et al. 1993) there were no significant differences between the efficiency of the dredges for the two larger size classes (90-109mm and 110mm + SL). The implications of this selectivity for stock assessment can be seen from Figure 2. On the basis of the dredge survey, the population appeared to be dominated by 3-year-olds (100-109 mm), while in reality 2-year-olds (80-89 mm) were much more abundant. Not surprisingly, the dredge survey did not catch many 1-year-old scallops either, but these were also rarely seen in the diver surveys. This could be due to poor recruitment in the previous year, but may also be due to their low visibility on the seabed (pers. obs). Diver surveys may therefore only be fully effective for *P. maximus* of two years or older.

For two of the by-catch species, *Cancer pagurus* and *Luidia ciliaris*, efficiency estimates from the depletion experiment were more than twice those from the diver surveys. This was a surprising result, until the effect of scallop dredging on the indirect mortality of by-catch species was considered (see also McLoughlin et al. 1991). As an extension of this study, damage to benthic organisms which encountered dredges without being captured, was also examined (Jenkins et al. 2001). On the basis of that study, 56% of *C. pagurus* left in the dredge tracks were expected to die (or be eaten) within a day of dredging (see also Hill et al. 1996, Veale et al. 2001). The respective figure for *L. ciliaris* was 31%. If it is considered that these additional individuals were effectively removed by dredging, values for total fishing mortality (catch plus indirect mortality) can be calculated. These work out as 67.0% for *C. pagurus* and 44.8% for *L. ciliaris*, very similar to the estimates of efficiency from the depletion experiment (68.5% and 47.5%, respectively). For the third species *Porania pulvillus*, none of the individuals left in the dredge track were expected to die (Jenkins et al. 2001). In this case, however, estimates from the depletion experiment and diver surveys were relatively similar (21.4% compared to 16.3%). Unlike the diver survey, the depletion experiment incorporated indirect fishing mortality into estimates of efficiency by measuring the decline in the abundance of live animals over the course of the study (10 days). It was therefore deemed appropriate to compare total fishing mortality from the diver surveys with estimates of efficiency from the depletion experiment. Indirect mortality would have had little effect on the depletion experiment results for *P. maximus*, as only 5% of individuals left in the dredge track were expected to die. The inability to calculate efficiency from the depletion experiment for the fourth by-catch species, *Asterias rubens*, was probably due to this species' high mobility and tendency to aggregate on dredge tracks (Kaiser and Spencer 1996, Ramsay et al. 1998, Veale et al. 2000a).

In summary, this study found that both depletion experiments and diver surveys of dredge tracks were effective for assessing scallop dredge efficiency. Differences between the results from the two methods were probably due to the inherent variability of dredge efficiency and emphasize the need for error terms to be built into efficiency estimates. The depletion experiment was more cost-effective (in terms of person hours) than the diver surveys, but provided little information on dredge selectivity. We therefore suggest that a combination of approaches is probably most appropriate.

ACKNOWLEDGMENTS

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PROBLEMS, PREDATORS, AND PERCEPTION: MANAGEMENT OF QUAHOG (HARDCLAM), *MERCENARIA MERCENRIA*, STOCK ENHANCEMENT PROGRAMS IN SOUTHERN NEW ENGLAND

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ABSTRACT Throughout southern New England and Long Island Sound, northern quahogs (*Mercenaria mercenaria* Linne), support large commercial and recreational fisheries. Municipal managers implement a number of tools to maintain the public stock, including enhancement with hatchery-reared juveniles, or seed. In 1999, we surveyed 68 municipal managers in the region (59 in Massachusetts and 9 from Long Island, New York) to identify the extent and magnitude of current programs and any obstacles to the success of such programs, specifically including predation. Of the 36 responses, 23 had used or were using *M. mercenaria* seed, planting an average of 1.4 million seed (± 355,000) at an average shell length of 15.9 mm (± 1.4) on a variety of substrates. Estimates of seed loss in the first year varied widely, but averaged 44% (± 6.2). Estimates of survival to market size were significantly higher ($P = 0.023$) in Massachusetts (49% ± 8.5) than New York (25% ± 4.0), the only significant difference between the two states. Losses were attributed to a variety of factors, but all respondents indicated predation was a major cause of that loss. Of predators identified as threats to *M. mercenaria* public seeding, green crabs, *Carcinus maenas*, were cited most frequently and scored as very serious predators (8.3 on a scale of 1 to 10 with 10 being the most damaging); other crabs, starfish, and gastropod predators were also perceived as serious threats. Managers were divided evenly on the effectiveness of predator trapping in reducing predator populations either within or among years in an attempt to protect seed. Our survey identified several areas where additional scientific study would benefit these municipalities: (1) assessment of seed survival to market size; (2) experimental demonstration of predators responsible for losses; and (3) evaluation of the effectiveness of trapping in both reducing predator populations and protecting seed.

KEY WORDS: public stock enhancement, *Mercenaria mercenaria*, *Carcinus maenas*, quahog, hardclam

INTRODUCTION

Throughout southern New England and Long Island Sound, northern quahogs, also known as hard clams (*Mercenaria mercenaria* Linne), support large commercial and recreational fisheries; for example, the commercial harvest in New York was over $17 million per year from 1995 to 1999 (pers. comm., National Marine Fisheries Service, Fisheries Statistics and Economics Division, Silver Spring, MD). Management of these fisheries, although subject to state and federal restrictions, is primarily carried out by municipal programs, which implement a variety of management tools to maintain the resource.

One strategy available to resource managers is to supplement the existing wild stock with hatchery-reared juvenile "seed" supplied by commercial and public shellfish hatcheries. Planting seed on public grounds may enhance the fishery directly, by introducing clams that survive and grow to a legally harvestable size, and indirectly by increasing larval supply and subsequent year classes. A more subtle benefit may be a dependable interannual supply of littlenecks and cherrystones, the two smaller and most valuable market size categories. Despite these possible benefits, quantitative tests of the success of such efforts are rare even in the largest, most ambitious programs (e.g., Macfarlane 1998). Predation, in particular, is recognized as a factor that may dramatically limit success of public seeding programs of various invertebrate species (e.g., Stoner & Davis 1994, Peterson et al. 1995, Barbeau et al. 1996). Similarly, predation is considered a major obstacle to success of private aquaculture of *M. mercenaria* in the northeastern portion of the United States (Spatz et al. 1996).

Despite this lack of quantitative evidence of benefits and concerns about predation, this method of stock enhancement has been widely adopted in the region, largely based on the qualitative perception that seeding benefits the fishery. Notably, the high incidence of the hatchery-reared subspecies *M. mercenaria* var. *notata* (which has a distinctive zig-zag shell coloration) in the catch is often cited by proponents of the method as evidence of success (W. Cameron Walton, pers. comm.).

Although the authors encourage a long-term rigorous assessment of the success of these public seeding programs, the reality is that many of these programs are driven by the perceptions of the local managers. Such perceptions are important not only because they steer management, but because they reflect many combined years of natural history observation and field work. In our study, therefore, we surveyed municipal shellfish managers throughout Massachusetts and Long Island, New York to: (1) describe the extent and nature of current municipal seeding programs; (2) identify potential losses of seed; and, in particular, (3) determine the extent and nature of loss of seed to predation.

METHODS

Our three-page survey (Appendix A) consisted of four sections: use of hatchery-raised *M. mercenaria* seed; handling of seed; losses of seed; and predation. Most questions were multiple-choice or required a quantitative answer. We indicated that survey participants would remain anonymous in the analysis of results. Draft versions were pilot tested with several shellfish constables on Martha's Vineyard, MA.

We mailed the survey on April 30, 1999 to all of the 59 Massachusetts shellfish constables listed with the Massachusetts Division of Marine Fisheries. One month later, we sent a duplicate of the survey with a second request to nonrespondents. Finally, two months later, we called nonrespondents requesting the return of a completed survey.

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In response to inquiries from municipal shellfish managers on Long Island, NY, on December 6, 1999, we sent surveys to nine municipal shellfish managers, whose names were provided by G. Rivarra of the Cornell Cooperative Extension. Unlike the Massachusetts recipients, the New York recipients were clustered geographically (toward the eastern end of the island). Follow-up requests were handled as they were with Massachusetts recipients.

In compiling results, some responses were simplified or standardized. For example, an estimated loss of 40–60% of planted seed was reduced to the median of 50%. In addition, if seed size was given as mesh size, we converted this to an average shell length. For identification of predators and their perceived effect, we asked respondents to list up to five of the worst predators upon seed. To quantify the threat posed, we asked further for respondents to score each predator with a number from 1 to 10, with 10 indicating that the predator was capable of inflicting severe losses. To capture both frequency of listing and perceived magnitude of the threat, we weighted each predator "vote" by its quantitative rating to derive a summed score. To test for differences between states, we conducted paired *t*-tests, assuming unequal variances between the populations with a Bonferroni adjusted probability.

RESULTS

Survey Response

Of the 68 surveys mailed, 36, or ~53%, were completed and returned. Of these responses, 23 respondents had used hatchery-reared seed as a stock enhancement tool, of which only one Massachusetts respondent had discontinued use after 1995. All nine respondents in New York currently use hatchery seed, and 13 of the 27 Massachusetts respondents currently seed. Several negative respondents (those who responded but indicated no current or past use of seed) indicated that their local shellfish beds were closed because of pollution, so they were implementing no stock management programs; whereas, two others expressed interest in implementing seeding in their municipalities.

Use and Extent of Hatchery Seed

Among affirmative respondents (Table 1), managers have used seed on average for 12.6 years, with no difference between states ($P = 0.99$). On average, Massachusetts municipalities received just under one million seed; whereas, New York municipalities received closer to two million, although this was not significantly different ($P = 0.194$). Programs varied widely in magnitude, from 50,000 to six million seed received in the most recent year. Typically, the size of the seed upon receipt was 5.2 mm in shell length (± 1.12, $n = 21$), with no difference between states ($P = 0.512$). Most respondents received seed in late spring/early summer.

Although two New York municipalities planted the seed immediately upon receipt, most managers maintained the seed for additional grow out, placing the seed in trays, rafts, or upwellers to obtain additional growth and protect them from predation. Seeding typically occurred when the seed were 15.9 mm (± 1.44, $n = 22$). Planting of these seed occurred from August to November. Several managers noted that they would occasionally receive seed in the fall and plant these directly. In addition, several managers based time of planting on the size of the seed; one constable in Massachusetts would keep small seed under netting through the winter to allow an additional year of growth before planting. Most planted the seed on unvegetated sand, mud, or muddy sand substrate (Fig. 1), although gravel and vegetated bottom were occasionally used.

Losses of Seed

Managers estimated that they lost 44% of planted seed within the first season (Table 1), with no significant difference between states ($P = 0.225$). Notably, estimates of first year loss ranged from 9 to 90% and tended to be lower in Massachusetts than New York ($P = 0.225$). Based on their observations, every respondent using seed indicated that predation was a major cause of that loss (Fig. 2). Losses attributable to the stress of handling and dispersion by currents were also cited; whereas, theft by private individuals was discounted as a potential source of loss (Fig. 2). Additional suggested sources of loss were: freezing temperatures (two respondents); the quahog parasite QPX (one respondent); and damage from raking (one respondent). There was no relationship between planting size and estimated first year mortality (Fig. 3). Despite these initial losses, managers estimated that 39% of planted seed survived to market size (Table 1). New York respondents, however, estimated a significantly lower survival rate of seed to market size than their Massachusetts counterparts ($P = 0.023$); 25 versus 49%, respectively. As with estimates of first year loss, estimates of survival to market size ranged widely, from 5 to 90%. Of those

TABLE 1.

Description of municipal *Mercenaria mercenaria* seeding programs in Massachusetts (MA) and New York (NY).

Factor	MA	NY	*P*	All Responses Combined
Years of use	12.6 (± 2.40, $n = 13$)	12.7 (± 1.55, $n = 9$)	0.986	12.6 (± 1.52, $n = 22$)
Quantity of seed received last year	994,429 (± 422,906.2, $n = 14$)	1,988,111 (± 598,288.4, $n = 9$)	0.194	1,383,261 (± 354,898.6, $n = 23$)
Average seed size upon receipt (mm)	4.6 (± 1.42, $n = 14$)	6.2 (± 1.85, $n = 7$)	0.512	5.2 (± 1.12, $n = 21$)
Average seed size upon planting (mm)	16.7 (± 2.35, $n = 13$)	14.8 (± 1.05, $n = 9$)	0.477	15.9 (± 1.44, $n = 22$)
Loss of seed upon planting	39.3% (± 7.91, $n = 13$)	54.6% (± 8.95, $n = 6$)	0.225	44.2% (± 6.19, $n = 19$)
Survival of seed to market size	48.6% (± 8.48, $n = 11$)	24.6% (± 4.03, $n = 7$)	0.023	39.3% (± 6.01, $n = 18$)

Each table value is followed by one SEM and the responding sample size.
The *P*-value indicated is the Bonferroni adjusted probability of differences between states.

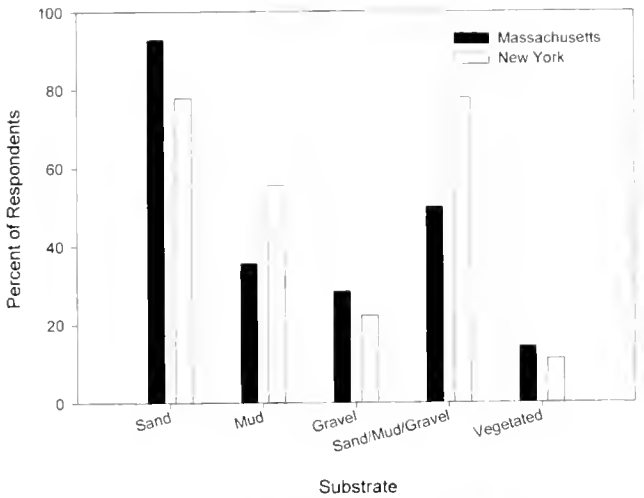


Figure 1. Use of various substrates for seeding of *Mercenaria mercenaria* by municipal programs in Massachusetts and New York.

managers planting seed < 25 mm shell length, there was a significant positive correlation between the average size of planting and the estimate of survival to market size ($P = 0.046$), excluding one outlier (Fig. 3).

Identifying and Controlling Predation on Seed

Many of the users of seed took measures to protect their seed from predation both before planting (85%, $n = 20$) and after planting (50%, $n = 22$). Predominantly, managers used mesh and netting to keep predators away from seed; a subset used traps in an effort to reduce the abundance of various predators. Of those who took any measures to protect seed, 62% felt that their efforts had had some success increasing seed survival, 14% felt that they had made efforts without much success, and 24% indicated that they had made no efforts. In addition, 46% of respondents had tried and given up various methods of protecting seed. Abandoned methods included: various barriers (nets, rafts, etc., $n = 5$), altered fishing practices (overwintering, closed areas, $n = 2$), direct predator reduction by trapping ($n = 2$), and biological control ("guarding" seed with toadfish, *Opanus tau*, to deter crab predation).

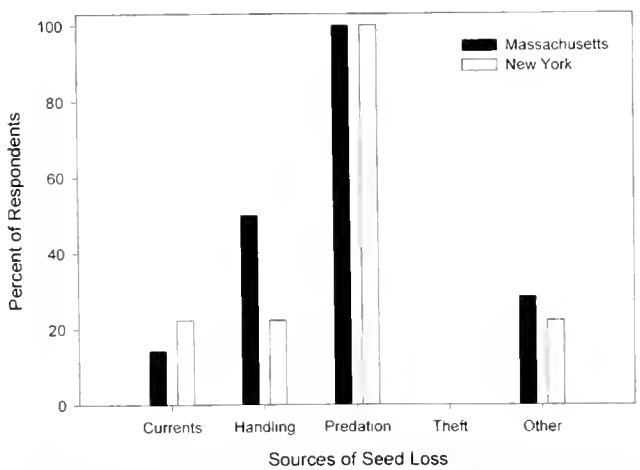
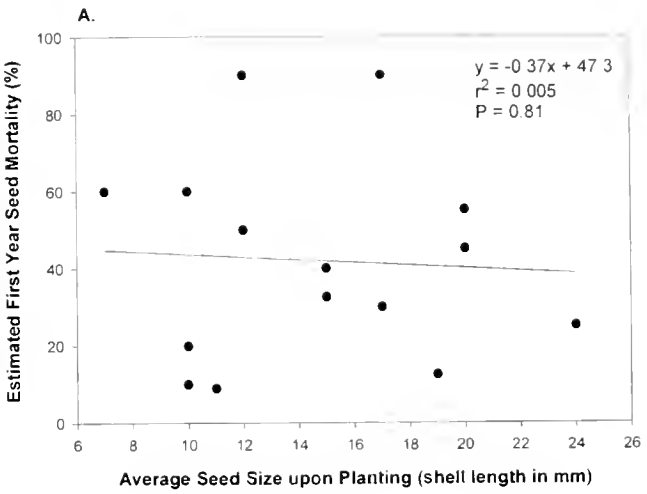


Figure 2. Various perceived sources of loss of *Mercenaria mercenaria* seed by municipal programs in Massachusetts and New York. See text for explanation of "other" sources of loss.



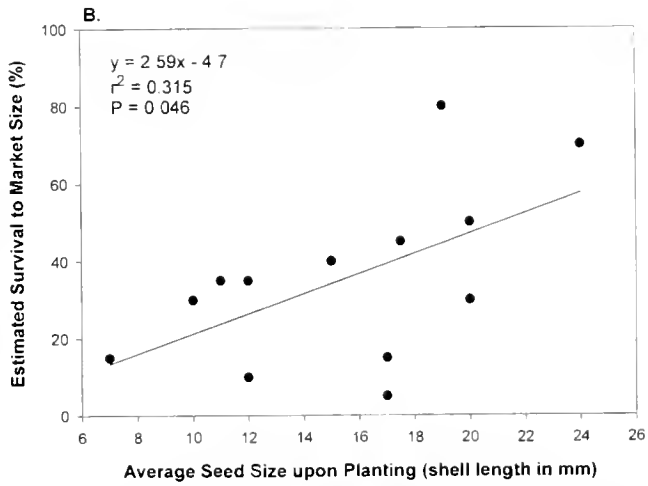


Figure 3. Relationship between average size of planting and A) estimated first year losses ($n = 15$) and B) survival to market size ($n = 13$) of *Mercenaria mercenaria* seed.

A wide variety of predators were recognized as threats to the success of municipal seeding programs (Fig. 4). Of the noted predators, green crabs (*Carcinus maenas*) were mentioned most often (15 of 22 respondents) and were considered capable of inflicting very serious losses (receiving an average score of 8.3 out of 10). Whelks (*Busycon carica* and *B. canaliculatus*) received the second highest summed score, which was approximately half the score of *C. maenas*. Two other portunid crabs were considered serious threats as well: the calico, or lady, crab (*Ovalipes ocellatus*) and the blue crab (*Callinectes sapidus*). Other predators considered serious threats by many respondents were (in order of scored importance): mud crabs (various xanthiid crab species), unspecified "crabs," starfish (*Asterias forbesi*), horseshoe crabs (*Limulus polyphemus*), spider crabs (*Libinia dubia* and/or *L. emarginata*), muricid drills (presumably *Eupleura caudata* and/or *Urosalpinx cinerea*), and sea gulls (various *Larus* spp). Other predators were noted by only one or two respondents, such as toadfish (*O. tau*), eels (presumably *Anguilla rostrata*), seals (presumably *Phoca vitulina*), sponges (unspecified *Cliona* spp.), and humans (*Homo sapiens*). Note, however, that only a single respondent listed rock crabs (*Cancer irroratus*) but gave them the highest

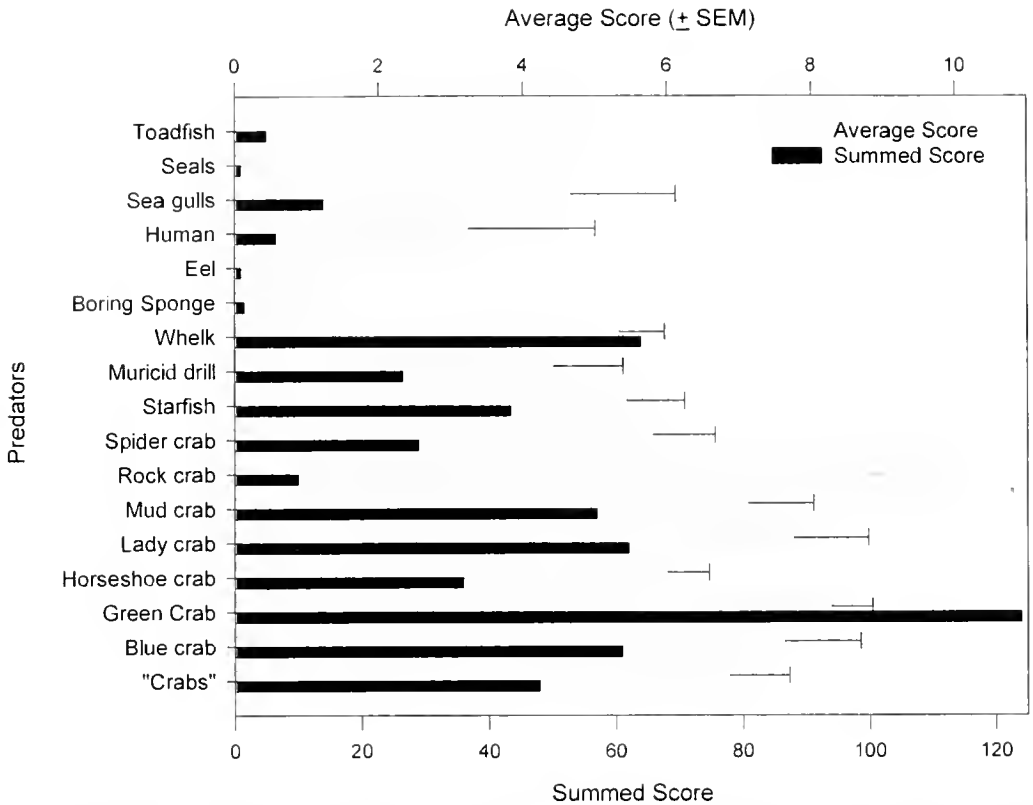


Figure 4. Perception of threat posed by various predators to the success of *Mercenaria mercenaria* municipal seeding programs in Massachusetts and New York. Predators were scored from 1 to 10, with 10 being the worst. Summed scores were derived by adding each of the scores together, reflecting both perceived threat and frequency of citation. See text for scientific names of species.

score possible, indicating the local importance of that particular predator.

Most managers (81%, $n = 21$) had noted changes in the predator community. Although responses varied, 10 respondents had observed a change in the crab community; of these, one had noted a decrease in green crabs, one indicated that the number of crabs varied, and eight had seen an increase in various portunid crabs. Conversely, six respondents had observed a change in the abundance of starfish; of these, one indicated an increase, one indicated that the population varied, and four had seen a decrease in starfish. Last, gastropod predators were seen to increase by two respondents.

In an attempt to reduce losses to predation, most respondents (65%, $n = 23$) had attempted or were currently trapping predators. Based on this experience (Table 2), almost half (44%, $n = 9$) of Massachusetts respondents felt that trapping noticeably reduced predator abundances across a season; whereas, most New York managers (75%, $n = 4$) observed intra-annual reductions. Fewer managers had opinions about the interannual effectiveness of trapping ($n = 10$), perhaps reflecting uncertainty. Of those who responded, respondents' opinions reflected the intra-annual perceptions; three of seven Massachusetts managers and two of three New York managers noted interannual reductions in predator abundance because of trapping. Those who supported trapping noted clear reductions in the abundance of the predators, and several were increasing the magnitude of their program. Those who did not trap or had given it up expressed various concerns, including: attraction of predators to the area, the large acreage involved, negative effects on nontarget species, including endangered species and a general sense of ineffectiveness (as one respondent wrote, "One can not defeat Mother Nature"). Two respondents specifically indicated a need for a test of the effectiveness of trapping before they could answer the question.

TABLE 2.

Perceived effectiveness of trapping in reducing predator populations by municipal *Mercenaria mercenaria* seeding programs in Massachusetts (MA) and New York (NY).

Factor	MA	NY	All Responses Combined
Observed intra-annual reductions	44.4% ($n = 9$)	75% ($n = 4$)	53.8% ($n = 13$)
Observed interannual reductions	42.9% ($n = 7$)	66.7% ($n = 3$)	50% ($n = 10$)

DISCUSSION

We were struck by both the response and degree of interest in regional public seeding of *M. mercenaria* var. *notata*; over half of the managers that were mailed surveys completed and returned the survey, an unusually high response rate in mailed surveys. Not surprisingly, a large percentage (64%) of those responding used seed to enhance their municipal stocks of *M. mercenaria*. If we conservatively assume that nonrespondents had not used seed, then approximately one-third of those municipalities surveyed (23 of 68) use or have used hatchery-reared seed; we know this to be an underestimate, because at least two municipalities that conduct

seeding did not complete their surveys (W. Cameron Walton, pers. comm.). Based on several notated answers, many respondents who did not use *M. mercenaria* seed did so because their shellfish beds were closed and/or inappropriate for raising quahogs (e.g., many areas north of Cape Cod). Several nonusers, however, expressed interest in initiating seeding programs. Of those responding and conducting seeding, many expressed interest both in the results of the survey and additional sources of information as a means of improving their programs.

Managers recognized a variety of factors that limit success of their seeding programs. All, however, agreed that predation was a causal factor (Fig. 2). Despite attempts by most managers to increase seed survival by growing seed to a larger, and, therefore potentially less vulnerable, size, estimates of losses of seed upon planting varied widely but averaged 44% (Table 1). Interestingly, although there was no relationship between average planting size (< 25 mm shell length) and perceived losses in the first year, there was a significant increase in perceived survival to market size with increasing planting size (Fig. 3). This lack of agreement in estimates suggests that either most losses occur after the first year (thus producing the positive correlation between planting size and survival to market size) or that managers have difficulty estimating first year survival. Because much ecological literature indicates the importance of prey size in determining prey vulnerability to crabs (see Juanes 1992 for a review), it seems likely that managers have not observed a positive relationship between planting size and first year survival simply because it is difficult to measure.

Between the states, there was a trend toward lower estimates of first year seed loss and significantly higher estimates of survival to market size in Massachusetts relative to New York (Table 1). Whether this difference reflects real differences in the field (e.g., the predator community, water quality) or between the attitudes of managers is unclear, but highlights the need for repeatable, rigorous field assessment of survival.

The identification of particular predators varied widely among responses, reflecting in part the temporal and spatial variation of predator populations (Fig. 4). Despite this variation, green crabs (a nonindigenous species originally from Europe) were cited by the largest number of respondents and were considered a very serious threat by most, receiving the highest summed score by far. Crabs, in general, were recognized as major predators, as were starfish and predatory gastropods. Interestingly, almost all respondents who commented upon changes in crab populations had observed an increase in portunid crabs, potentially increasing the perception of damage done by these species.

Despite agreement upon both the importance of predation and the green crab as the most damaging predator, municipal managers were divided on the effectiveness of predator trapping, including a number who were unable or unwilling to assess trap effectiveness. Of those who expressed an opinion on the success of trapping either within or across years, about half found trapping effective. There was a tendency (Table 2) for New York managers to find trapping more effective than their Massachusetts counterparts, despite the countertendency for New York managers to perceive higher losses of seed (Table 1). This suggests several competing alternatives: (1) trapping may reduce predators but not increase seed survival; (2) trapping may reduce predators but absolute losses to predation are greater in New York municipalities; or (3) despite managers' perceptions, trapping does not noticeably reduce predators. The first alternative could be generated by several mechanisms: low seed survival despite trapping could be caused by factors other than predation, trapping of a predator other than the one responsible, and/or compensatory predation (either intra- or interspecific) after removal of the primary predator. The second alternative could be tested by conducting prey outplants in both regions to determine relative predation pressure. The last alternative requires a rigorous field test of the efficacy of trapping as a predator control technique.

Are the current seeding programs large enough to be economically significant within the region? Responding managers planted just under 32 million seed in the year immediately before receiving the survey. A very rough calculation of the relative importance of seeding upon *M. mercenaria* fisheries in these two states can be obtained by making various assumptions about survival and market prices. Based on the Massachusetts estimates of seed survival to market size (49%), approximately 6.9 million of the 14 million planted seed would survive to market size. If most of these are caught (80% given that their locations are known) during the first year of obtaining legal size, 5.5 million would be harvested. At a market price estimated $0.20/clam, municipalities might expect to generate ~$1.1 million by seeding (without considering any benefits of such stock enhancement upon subsequent recruitments). In New York, on the other hand, the lower estimate of survival to market size (25%) means that only 4.5 million of the 18 million planted seed would survive to market size. Making similar assumptions about harvesting and price, 3.6 million quahogs would be harvested in the first year, yielding a value of ~$720,000. Many of these seeded clams, however, may be harvested by recreational fishermen, and, thus, do not contribute directly to the regional economy. In addition, such calculations do not take into account any indirect benefit of seeding upon population abundance via reproduction.

In conclusion, municipal managers who have adopted seeding as a management tool recognize a variety of factors that limit survival of seed. Despite these losses, which vary in space and time even within a municipality, managers who used seed expressed strong belief in the effectiveness of seeding. The results of this survey suggest, however, several areas in which managers would benefit from additional information. First, programs would benefit from a quantitative assessment of survival of seed to market size, allowing a more accurate assessment of the worth of such enhancement efforts. Second, it is essential to determine experimentally the most important predators upon *M. mercenaria* seed to ensure that any predator removal efforts are targeting the correct species. Last, managers need an evaluation of the effectiveness of trapping. Managers themselves are unsure as a group, and, furthermore, the discrepancy between estimated seed survival and trapping effectiveness by New York managers suggests an area worthy of investigation.

ACKNOWLEDGMENTS

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Appendix A

Southern New England & Mid-Atlantic Shellfish Management Survey: Use of Hatchery-Reared Quahogs

Please fill out and return in the SASE provided, or mail to Bill Walton, Beals Island Regional Shellfish Hatchery, PO Box 83, Beals, ME 04611

I. Use of Quahog Seed

Do you (or your town) currently, or have you in the past, used hatchery-raised quahog seed to enhance your fishery?

___ Yes, currently use seed (indicate number of years in use ___)

___ Yes, used seed in the past but have stopped (indicate last year used 19__)

___ No, never used quahog seed (You have completed this survey and thank you for your participation. PLEASE RETURN THIS IN THE SASE)

II. Handling of Seed

1. In the last season you used seed, what was the approximate quantity of seed you received and/or produced?

_____

2. In the last season you used seed, what was the average size (shell length or mesh size) of the seed when you received it?

__ mm or mesh size

3. In the last season you used seed, what was the average seed size when you planted them out?

__ mm or mesh size

4. What is a rough time line of quahog seed in your possession in a typical year (for example, get seed in July and plant immediately, or get seed in July, put in floating rafts until October, and then plant out)

_____

_____

5. What type or types of bottom do you plant out on? (Check all that apply)

___ Sand ___ Mud ___ Gravel ___ Sand/Mud/Gravel Mix ___ Vegetated

III. Loss of Seed

1. What percent (estimated) of the seed you've planted out during a typical season did you lose?

   ___ %

2. What were the main causes of that loss?

   _ Normal effects of transportation/handling Theft

   _ Washed away by currents Predation

   _ Other (please specify) _____

3. In your overall experience, estimate typically what percent of seed you plant out makes it to market size in your town.

   ___ %

IV. Controlling Predation on Seed

1. If you did not immediately plant out the last seed you received, what attempts, if any, did you make to protect them from predation prior to planting?

   _____

2. Once you planted out your last seed, what attempts, if any, did you make to protect those seed from predation?

   _____

3. Please identify up to the 5 worst predators of your seed and enter them below. For each predator you mentioned, please rate it as a threat to your seed (10 = capable of inflicting severe losses upon seed; to 1 = very little threat).

   Predators: _____ Rating (10 to 1):

   Worst: _____ _____

   2nd worst: _____ _____

   3rd worst: _____ _____

   4th worst: _____ _____

   5th worst: _____ _____

4. Have you made any efforts to protect seed that have significantly increased seed survival?

___ Yes, have had some success ___ Made efforts without much success

___ No efforts made and or required

5. Are there any methods of protecting seed that either you or your town's prior constables tried but gave up? If so, what were they, and why were they given up?

No

__ Yes (please specify methods and reason for giving them up)_____

_____

6. Have you noticed any changes in the predator community in your local waters (for example, have starfish increased dramatically over the last decade)?

___ No

__ Yes (please specify those changes)_____

_____

_____

7. Among other things, I'm gathering information on the effectiveness of predator trapping. If you have trapped, or currently do so, do you think it noticeably reduces the predator population you're trapping either:

a) across a season ___ Yes ___ No

b) over consecutive years ___ Yes ___ No

Please comment on your experience with trapping predators to protect shellfish, in particular quahog seed.

_____

_____

You have completed the survey. Thank you very much for your assistance with this matter. Please return the survey in the provided envelope. I will be glad to make the results of the survey, as well as results of my research into ways of improving seed survival, to all respondents. If you would like to receive this information, please include your business card or your name and address on a separate sheet of paper. Again, thank you for your time and assistance.

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EFFECTS OF CAGING ON RETENTION OF POSTLARVAL SOFT-SHELLED CLAMS (*MYA ARENARIA*)

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ABSTRACT Postlarval transport is an important process affecting the population dynamics of many coastal benthic species. The first growth season after settlement represents one of the most influential life-history stages of many benthic species, including the commercially important soft-shelled clam *Mya arenaria*. In the present study, the effect of a mesh cage on the retention of postlarval soft-shelled clams was investigated using a laboratory flume. Three size categories of clams were exposed to a range of velocities both with and without the cage. The presence of the cage did not significantly increase the number of clams retained for most clam sizes and velocities tested. Only at the fastest velocity tested did the cage significantly increase the proportion of clams retained. Characterization of flow patterns indicated that flow velocities were reduced inside the cage at the fastest velocity but not at the lower velocity. We propose that the cage effects on retention are negligible in flows up to a critical velocity, above which they protect clams from resuspension. These results can be applied to field applications of mesh cages to optimize the beneficial caging effect.

KEY WORDS: *Mya arenaria*, postlarvae, cage, transport, resuspension, shear velocity, flume

INTRODUCTION

Harvesting of the soft-shelled clam, *Mya arenaria* L., is an important commercial industry along the coast of New England. Adult *M. arenaria* are collected from hundreds of coastal embayments from Canada to Cape Hatteras, producing a total annual revenue in excess of $12 million (U.S. Fisheries Report 1998). Yet the productivity of soft-shelled clam fisheries is erratic because *M. arenaria* recruitment is subject to extreme temporal and spatial variability (Beukema et al. 1978, Möller and Rosenberg 1983). Because adult population density is linked to recruitment, this variability results in dramatic population changes from season to season and among locations within the local habitat.

The observed variability in recruitment could result from changes in any one or more of the numerous processes operating on larval and postlarval stages. Local recruitment is a result of the following: initial larval supply, proportion of larvae that settle, post-settlement mortality due to predation and/or inadequate resources, and post-settlement transport processes. Although numerous studies have investigated the factors controlling initial larval settlement and mortality (Pfitzenmeyer 1962, Günther 1992, Rodriguez et al. 1993), few have examined the effects of hydrodynamic transport of postlarvae (Emerson and Grant 1991). Both physical processes and behavioral responses, such as depth of burrowing and byssal drifting, contribute to postlarval transport of recruits (Sigurdsson et al. 1976, Newell and Hidu 1986). In addition, over the first growth season, changes in size and burial depth could affect the proportion of clams retained in particular flow regimes. The physical parameter most relevant to hydrodynamic transport of recruits is bottom shear stress, because it controls the suspension and deposition of particles. Shear stress is defined as shearing strain caused by water moving over the bottom and is proportional to the rate of deformation of the fluid (Gerhart and Gross 1985).

In response to the high variability in *M. arenaria* recruitment, some fisheries have implemented programs to increase settlement and decrease resuspension of either "wild" postlarvae or cultivated spat by manipulating benthic conditions. One of the most successful techniques involves placing mesh netting (cages) over the sand flats to alter the hydrodynamics and exclude predators (Beal 1993, Marcotti and Leavitt 1997). Although the beneficial role of predator and disturber exclusion has been demonstrated (Beal 1993, 1994, Dunn et al. 1999), little is known about the mechanisms by which cages may alter the hydrodynamic regime and allow for enhanced population densities.

The goal of the present study is to quantify the effect of caging on the following: near-bottom flow characteristics and postlarval clam retention over a range of clam sizes and benthic shear stresses (as quantified with shear velocity u_*). We use a recirculating flume to simulate tidal flows common in the field and to quantify the effect of the cage on clam retention in a test bed of sediment. We define retention as the proportion of clams not eroded from the sediment in the presence of flow and resuspension as the quantity of clams eroded from the sediment and transported downstream. We focus on resuspension because of its relevance to natural situations where clam larvae or postlarvae dropped from suspension and settled in quiescent periods of a tidal cycle, but are subjected to resuspension during vigorous periods of flood and ebb. The flume studies also are relevant to aquaculture situations where clam spat are seeded at low tide and exposed to suspension during subsequent tidal excursions. Our research addresses the following questions:

How does the cage affect near-bottom velocities and turbulence in the flume?
How does the cage affect postlarval transport and retention of *Mya arenaria*?
How do these caging effects vary with clam size (i.e., for recruits during their first growth season) and with flow velocity (i.e., over a range of realistic shear stresses) from a tidal environment?

We expect the cage to alter near-bottom flows by reducing the benthic shear stress under the structure. For a specific clam size, this reduction in shear stress should result in a reduction in numbers of clams resuspended, at flows that typically would erode uncaged buried clams. Because higher shear stresses typically are

required to resuspend larger clams (Emerson and Grant 1991, Dunn et al. 1999), it is important to conduct our flume studies over a range of clam sizes and flow speeds, in order to provide results that can be generalized to the field.

This study is intended to provide a mechanistic understanding of how mesh caging influences resuspension of *M. arenaria*, which represents a potentially important loss to a local population. By focusing on this mechanism, we leave unexplored other potential cage effects such as those on larval settlement and deposition; mortality due to predation; and enhancement or depletion of food supply. However, a characterization of the flow alterations caused by caging provides a reference point for predictions of other hydrodynamically-controlled processes such as deposition and food flux.

MATERIALS AND METHODS

Test Organism and Sediment

Flume experiments were conducted in 1997 and 1998 to test the effects of caging on retention of three size- categories of clam recruits (corresponding to a progression of first season sizes of clams resulting from a late spring settlement in Barnstable Harbor, Massachusetts; data not shown) over a range of shear velocities. The shell length (SL) of the small, medium, and large size recruits was measured as 1.3 ± 0.17 mm (mean ± st. dev.; n = 100), 1.8 ± 0.15 mm, and 2.3 ± 0.3 mm, respectively. Flume studies were conducted in the summer of 1998 and spring 1999 to characterize flow along the flume and around the cage. Postlarval *M. arenaria* were acquired from Beals Island Regional Shellfish Hatchery, Beals, Maine in 1997 and from Mook Sea Farm, Inc., Walpole, Maine in 1998.

All clams were held in plastic containers with 64 μm mesh tops and supplied with 10 μm filtered, running seawater (~21°C) from Vineyard Sound. In 1997, clams were fed Tahitian *Isochrysis galbana* at least once a day, but cultures experienced higher mortality than expected. Thus, in 1998, the clams were fed twice a day with T. *Isochrysis galbana* and *Tetraselmis* sp. (both species from Instant Algae, Reed Mariculture Inc.) and aerated the holding containers to improve survival rates.

Sediment used for the experiments was collected from Barnstable Harbor. Only the surficial, oxic layer of sediment was used to avoid hydrogen sulfide toxicity and provide suitable substrate for the clams. For consistency among experiments, all sediment was passed through a 2 mm sieve and retained on a 180 μm sieve. Based on extensive sampling, over 65% of Barnstable Harbor sediment was within the 180 μm to 2 mm size range (dry wt. basis); therefore, this test sediment was a reasonable approximation of field conditions (authors' unpublished data).

Sediment was kept frozen at −4°C until use. After thorough mixing, defrosted sediment was placed in the sediment box and the surface was leveled. Prepared sediment had a median grain size of 150 to 180 μm.

Flow Characterization and Cage Setup

Experiments were performed in a recirculating flume at the Woods Hole Oceanographic Institution's Rinehart Coastal Research Center. The flume consisted of a 30 cm deep, 60 cm wide, and 17 m long channel through which water was circulated by an impeller pump (Butman and Chapman 1989). Flow was rectified at the headbox of the flume by a Plexiglas grid of 1.2 cm squares 7.5 cm long. At the very downstream end of the flume, an accordion weir set the area available for water outlet. Simultaneous alongstream and vertical velocities were measured by a two-axis Laser Doppler Velocimeter (LDV) (Trowbridge et al. 1989). Velocity profiles were measured 1.7 m upstream of the test section (Fig. 1). Shear velocities (u_*) were estimated from the flow profiles using an iterative method to calculate u_* in a steady, one-dimensional open-channel flow (Trowbridge et al. 1989). To ensure that the desired velocity was produced, water velocities were measured at 25 hertz and averaged over 4 minutes of measurements at each of 10 heights above the flume bottom (0.9, 1.2, 1.6, 2.1, 2.7, 3.4, 4.2, 5.0, 6.0, and 8.0 cm). These velocity profiles were conducted for all trials to estimate variation in flow among replicates for specific target velocities (target velocities ranged from u_* = 0.7–2.0 cm s^{-1}).

The working section of the flume was 12.5 m downstream from the water entrance. At this distance the turbulent boundary layer was fully developed. For these experiments, a section of the flume's bottom was replaced with a removable acrylic tray (55 × 55 cm) containing a central recessed sediment box (20 × 20 × 2 cm deep) (Fig. 1). The cage (described below) surrounded the sediment box and was secured to the acrylic tray. For each experiment, fresh sediment was placed in the box and the surface leveled before fitting the tray into the flume. The edges of the tray were flush with the flume bottom. The water depth in the flume was approximately 12 cm and water temperature was 20°C to 22°C. We selected a target water depth of 12 cm to maintain a water column width-to-depth ratio of at least 5, thereby maximizing the accuracy of the shear velocity measurements (Nowell and Jumars 1987, Butman and Chapman 1989).

In all experiments, flow was established by adjusting the pump speed and outlet weir setting to attain the target water depth (h = 12 cm at a distance of 21 cm upstream of the test section; Fig. 1) and target free-stream velocity (u_8 = free-stream flow at 8 cm above bottom measured 1.7 m upstream of the test section). Once the target velocity and depth were achieved (within 5–10 minutes of introducing the tray), velocity profile measurements were initiated. Velocity profiles were reproduced with only slight variation (<5%) among replicates for a single target velocity. Afterwards,

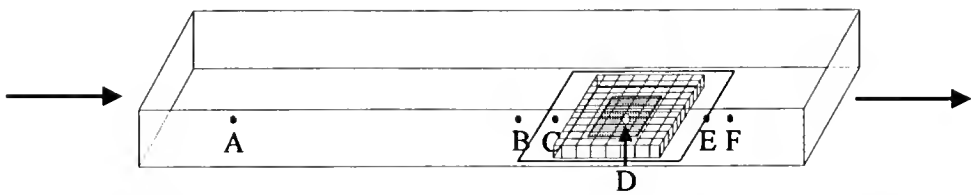

Figure 1. Oblique view of the test section of the flume showing the location of the cage and positions of velocity profile measurements. Respectively, A, B, and C are 170, 21, and 6 cm upstream of the cage front. D is 27 cm downstream from the leading edge of the cage. E and F are 10 and 20 cm downstream of the cage. Gray box within the cage represents the sediment box.

the flow speed was gradually reduced from the target velocity to u_8 = 3 cm s^{-1} and the tray was removed from the flume.

The cage used in these experiments was a 46 cm long × 54 cm wide × 5 cm high metal frame covered on the top and sides with plastic mesh netting of a size commonly used by aquaculturists and fishery managers (InterNet Inc. Minneoplis, MN; oriented flexible square; mesh size = 0.32 cm). In an ideal setup, the water depth should be at least 3 times the cage height in order to avoid generating free-surface interactions (Nowell and Jumars 1984). For our experiments, we were limited to a water depth of 12 cm and to a cage height of >4 cm to create adequate separation between the bottom of the flume and the roof of the cage. Although the flows above the cage were likely influenced by the structure's proximity to the free surface, the magnitude of this interaction, and its potential effect on bottom shear stress, were expected to be minor. This expectation was evaluated during the experiments using water height and velocity measurements. The cage support rods were positioned >5 diameters from the sediment box and had no detectable influence on water flow or sediment transport (Nowell and Jumars 1987).

At least one representative upstream shear velocity (u_*) was measured for each flow regime tested (Table 1). For most flow regimes, u_* was calculated during each experimental trial, and averaged across the 3 or 4 replicate trials to characterize the flow treatment (note values for u_* were not recorded in some trials due to technical problems). Shear velocities were selected to fall within the range of typical estuarine and bay flows, as derived from current meter data from Barnstable Harbor. At three sites in Barnstable Harbor, the range of velocities measured during a spring tide in 1998 corresponded to shear velocities of 0–3.8 cm s^{-1} and, over half of the time, flows were within the range used in these trials (0.7–2.0 cm s^{-1}) (authors' unpublished data).

Flow patterns around the cage were quantified by taking centered velocity profiles 6 cm upstream of the cage, mid-cage, and both 10 and 20 cm downstream of the cage (Fig. 1). Velocity profiles were also taken at these locations without the cage present. This array of profiles was conducted at shear velocities of u_* = 1.8 and 2.0 cm s^{-1}. These velocities were selected after conducting the clam retention trials, to investigate flow patterns at and below a shear velocity that yielded significantly greater clam retention in the caged treatment (See Results, Retention section). Flow deviations caused by the cage were quantified in the two flow regimes by calculating the time-averaged velocity at each measurement point and comparing the difference between the cage and no-cage profiles at both velocities. Fluorescein dye visualizations were performed to describe qualitatively the turbulence and flow separation generated by the mesh cage.

TABLE 1.

Mean alongstream velocities at 8 cm above flume bottom (u_8) and mean shear velocities (u_*) for all three clam sizes and flow treatments. Standard deviations are in parentheses.

Clam category	u_8 (cm s^{-1})	u_* (cm s^{-1})	n
Small	14.3 (0.3)	0.66 (0.01)	8
	30.1 (0.3)	1.30 (0.02)	8
Medium	32.6 (0.8)	1.41 (0.02)	4
	38.1 (0.5)	1.61 (0.02)	6
	40.1 (0.7)	1.73 (0.01)	6
	43.8 (0.4)	1.83 (0.01)	6
Large	47.2 (1.2)	1.97 (0.09)	6

Retention Trials

The main objective of the retention trials was to determine the effect of caging over a realistic range of field flows and first season clam sizes. Three or four replicate trials were conducted with and without the cage for each clam size over a range of selected velocities (n = 3 for all trials except when u_* = 0.7 and 1.3 cm s^{-1}). The small clams were exposed to shear velocities of 0.7 cm s^{-1} and 1.3 cm s^{-1}; medium size clams to 1.4–1.8 cm s^{-1}; and large clams to 2.0 cm s^{-1}. This experimental setup did not follow an ideal 3-factor design with caged and uncaged treatments in all combinations of flow speed and clam size for two reasons: the number of first season clams was limited and some data were incorporated from experiments conducted as a component of another study. The large clam data and part of the medium sized clam data (u_* = 1.4 and 1.6 cm s^{-1} trials) were gathered as part of a caging study we conducted in association with flume trials in Dunn et al. (1999).

In order to use the available clams most efficiently, we selected what we anticipated to be the most relevant flow speeds for each clam size. Lower speeds were used for smaller sizes because lower shear velocities were anticipated to erode smaller clams. The velocity ranges selected for each clam size were chosen based on preliminary flume experiments that determined the critical velocities needed for initial movement of sediment and clams, both buried and unburied. The lower velocities were selected to erode non-burrowing clams, but not significantly erode the sediment, while the higher shear velocities were intended to erode a portion of the sediment and thereby allow assessment of the burrowing clam's response to elevated flow.

For each retention trial, we selected 200 active clams. This number corresponds to a density of 5,000 clams per m^2 (within the 20 cm × 20 cm sediment box) and approximates actual Barnstable Harbor field densities (authors'unpublished data). The clams were suspended in seawater and then rinsed through a plastic cylinder (10 cm diameter × 20 cm high) held 1 to 2 cm above the sediment surface. Suspending the clams ensured that they were distributed evenly on the sediment. Use of the cylinder ensured that the clams settled onto the sediment box surface, not the acrylic perimeter. The clams were given 20 minutes to burrow (sufficient time for burial based on previous observations). After the burial time, the sediment tray was removed from the seawater table, the number of non-burrowing clams was recorded and those remaining on the sediment surface were removed. The tray was inserted into the flume under conditions of very low flow (u_8 = 3 cm s^{-1}) and made flush with the adjoining flume bottom. For the cage treatments, the cage and its supports were secured to the tray at this time. All trials were conducted for 40 minutes except for u_* = 1.7 and 1.8 cm s^{-1} which ran for 10 minutes because these were part of another study.

For all runs, the sediment remaining in the sediment box after the experiment was sieved on a 500 μm sieve and the fraction retained was either sorted for clams immediately or preserved in 80% ethanol, stained with Rose Bengal, and sorted later. In order to ensure that we were not losing clams during sediment transfers, we conducted procedural controls using the medium size clams. Procedural controls followed the same procedure as the retention runs except that the tray was placed in the water table instead of the flume. These controls showed that an average of 99% of seeded

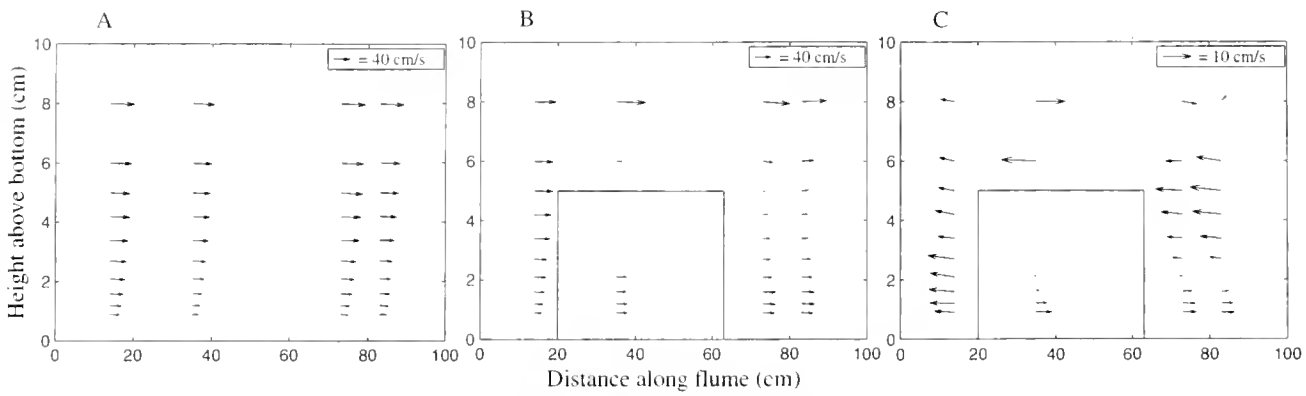

Figure 2. Vertical profiles of flow velocity (u) along flume with upstream shear velocity of $u_* = 1.8$ cm s^{-1}. A) Profiles without the cage (u); B) profiles with the cage (u_{cage}); C) deviations in velocity caused by the cage [i.e., $u_{cage} - u$]. To emphasize the vertical pattern, only the components of the vectors > 27 cm s^{-1} are graphed for profiles A and B. In profile C, deviations are true differences. Distances along the flume are relative and start 20 cm before the leading edge of the cage.

clams were recovered, indicating that we were not losing clams while transferring the tray from the seawater table to the flume.

For retention trials, results were analyzed separately for each clam size, with cage (two levels, present and absent) and velocity as fixed factors in two-way analysis of variance (ANOVA) (Systat version 6.1). The dependent variable was the percentage of clams retained. For the medium size clam results, two separate two-way ANOVAs were performed due to different times of exposure to flow (10 and 40 minutes) and for large-sized clam results a one-way ANOVA for cage effect was performed. Visual inspection did not reveal any pronounced deviations from a normal distribution. Variances were tested and found to be homogeneous (Cochran's test; $p > 0.05$).

RESULTS

Cage Effects on Flow

A comparison of velocity profiles conducted with and without the cage indicates that the cage altered near-bed flow at both $u_* = 1.8$ cm s^{-1} (Fig. 2) and $u_* = 2.0$ cm s^{-1} (Fig. 3). Without the cage, velocities fit the expected log-linear profile, and changed little (<8%) with downstream distance (Figs. 2A, 3A). The presence of the cage disrupted this pattern by changing flows inside the cage, just above the upper rim of the cage's frame and just downstream of the trailing edge of the frame (Figs. 2B, 3B). Dye visualizations revealed that the leading face of the cage caused flow separation around the leading cross-sectional portion of the frame. The dye revealed eddies shedding off the upper edges of the cage and contributing to the observed increase in turbulence above and downstream of the cage. The velocity profiles indicated that mean velocities were reduced downstream of the cage at $u_* = 1.8$ cm s^{-1} and both within and downstream of the cage at $u_* = 2.0$ cm s^{-1} (Fig. 2C, 3C).

The effect inside the cage was different at $u_* = 1.8$ cm s^{-1} than at $u_* = 2.0$ cm s^{-1}. Flow deviations (Fig. 2C, 3C) indicated that velocities at $u_* = 1.8$ cm s^{-1} were increased inside the cage (at heights of 0.9, 1.2, 1.6 cm), yet at $u_* = 2.0$ cm s^{-1} these velocities were consistently reduced inside the cage. The vertical patterns of flow alteration were different at the two velocities. At $u_* = 1.8$ cm s^{-1} the flow deviations became less pronounced with height off of bottom, but at $u_* = 2.0$ cm s^{-1} the flow deviations became more pronounced with height off of bottom. These patterns suggest that the cage alters the flow most strongly at the sediment interface at $u_* = 1.8$ cm s^{-1} and at a height above the sediment interface at $u_* = 2.0$ cm s^{-1}.

The cage did have a small but measurable effect on water height in the flume. Upstream water heights along the flume increased in the presence of the cage, while downstream water heights decreased in comparison to no-cage heights at comparable target velocities. Water heights were elevated approximately 7% just in front of the cage (position C in Fig. 1) yet at the positions of our water height measurements and velocity profiles the increase was <3%. From the trailing edge of the cage to 3 m downstream, the heights were decreased by about 3% as compared to the no cage water heights. For the flow profiles at $u_* = 2.0$ cm s^{-1} and $u_* = 1.8$ cm s^{-1}, water heights over the cage were increased 4% and 2%, respectively. These water height deviations are relatively small and probably do not have a noticeable effect on flows within the cage. The proximity of the free-surface of the water to the upper face of the cage, however, may constrain the flow over the top of the cage.

Retention Trials

The cage tended to enhance retention of clams at some, but not all, combinations of clam size and shear velocity (Fig. 4). For small clams, slightly more clams were retained in cages, but the result was not statistically significant (Table 2). With medium size clams, there was no clear pattern of cage effect; neither treatment consistently retained more clams (Fig. 4). The only significant increase in clam retention with the cage ($p = 0.046$) occurred with the large clams at the greatest shear velocity used, $u_* = 2.0$ cm s^{-1} (Table 2). Potentially significant differences for the smallest category of clams could have been obscured by the high variability in results. On average, fewer than 8% of clams placed on the sediment box did not burrow.

Within a clam size, more clams tended to be eroded at higher velocities, but this trend was significant only for medium size clams for the comparison of $u_* = 1.7$ and 1.8 cm s^{-1} (Table 2). We had anticipated a direct relationship between shear velocity and clam erosion based on the study of Dunn et al. (1999). For our medium size clams, the relationship between velocity and clam

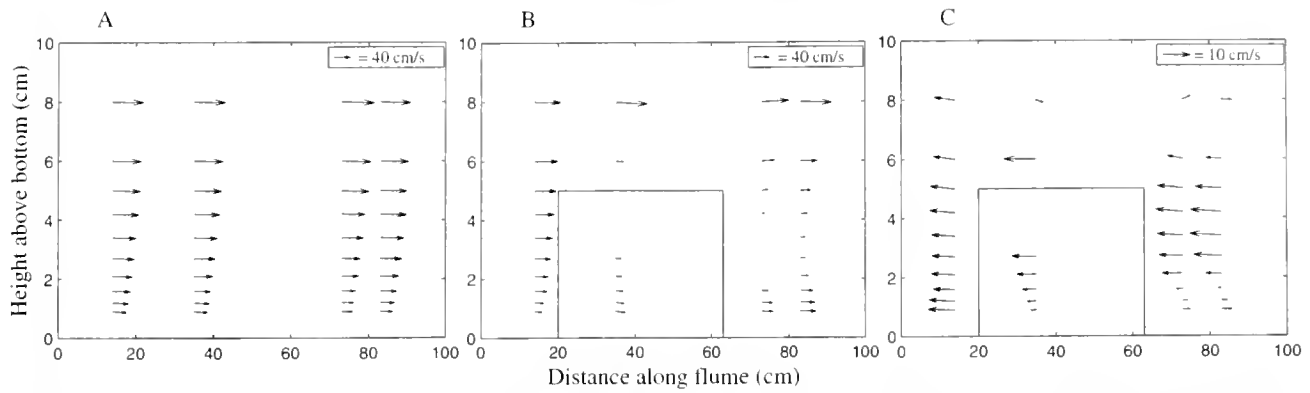

Figure 3. Vertical profiles of flow velocity (u) along flume with upstream shear velocity of $u_* = 2.0$ cm s^{-1}. A) Profiles without the cage (u); B) profiles with the cage (u_{cage}); C) deviations in velocity caused by the cage [i.e., $u_{cage} - u$]. To emphasize the vertical pattern, only the components of the vectors > 27 cm s^{-1} are graphed for profiles A and B. In profile C, deviations are true differences. Distances along the flume are relative and start 20 cm before the leading edge of the cage.

retention was not linear but appeared to have some critical velocity above which there was a notable reduction in clam retention.

DISCUSSION

Cage Effect on Clam Retention in the Flume

The most consistent result from our flume studies was the lack of a significant cage effect on clam retention in most flow regimes. This result was a surprise because the presence of a mesh structure along a flow path is known to alter flow patterns (Hulberg and Oliver 1980, Nowell and Jumars 1984), and natural mesh-like obstacles such as marsh grasses have been observed to reduce flow velocities by two to 10-fold (Gambi et al. 1990, Leonard and Luther 1995). A reduction in flow velocity was expected to occur in the cages and result in enhanced clam retention. The lack of significance did not appear to be due solely to low power of the statistical tests, as an increase in the number of replicate trials per treatment (from 3 to 4) did not result in higher significance (n = 4 for target shear velocities of $u_* = 0.7$ and 1.3 cm s^{-1}).

The one treatment that did show a significant effect of the cage on retention was the combination of the highest flow velocity ($u_* = 2.0$ cm s^{-1}) with the large clam size (SL = 2.3 mm). We

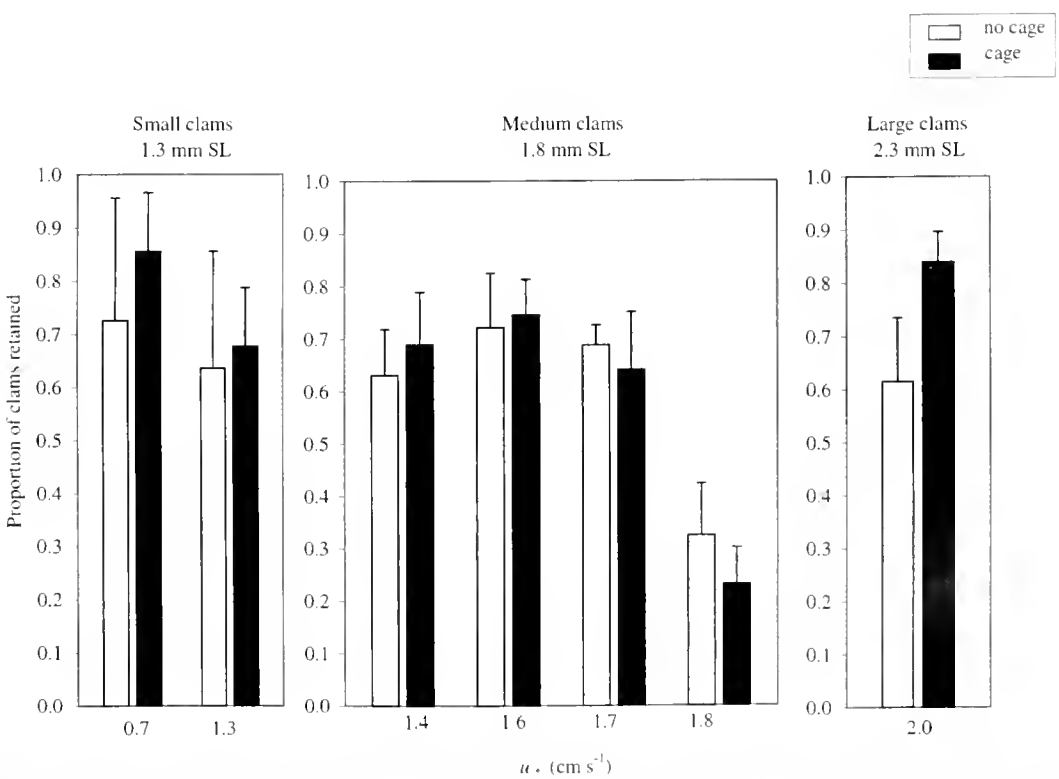

Figure 4. Mean proportion (±1 S.D.) of clams retained over a range of shear velocities with and without cage treatments. N = 3 for all runs except $u_* = 0.7$ and 1.3 cm s^{-1} where n = 4.

suggest that the mechanism for this cage effect is a substantial reduction in flow velocities within the cage in this particular flow regime. Reduced flow velocities should reduce the shear stress exerted on the sediment surface, thereby decreasing the rate of erosion of sediment and clams. This relationship between shear stress and erosion was observed in the present study for the medium size clam treatment at u_* = 1.7 and 1.8 cm s^{-1} (significant effect of velocity in Table 2), and has been reported by Roegner et al. (1995) and Dunn et al. (1999). We suspect that the absence of a significant cage effect on clam retention in flows slower than u_* = 2.0 cm s^{-1} was due to the failure of the cage to reduce boundary shear stress in these conditions.

Flow measurements recorded within the cage in the fastest oncoming flow treatment (u_* = 2.0 cm s^{-1}) and a slower flow (u_* = 1.8 cm s^{-1}) support our explanation for the clam retention results. The cage caused a measurable decrease in velocities near the sediment only in the fastest oncoming flow (Fig. 3). One plausible explanation for this effect is that the cage was starting to act more as a bluff body at the higher flows, blocking flow through the mesh and forcing the water up over the upper cage surface. This effect of producing a 'skimming flow' has been predicted and demonstrated for a sufficiently dense array of tubes or cylinders in boundary-layer flows (Morris 1955, Eckman et al. 1981, Nowell and Jumars 1984). We speculate that a comparable process occurs in our cage at oncoming flows of u_* = 2.0 cm s^{-1} and higher: in this flow regime, the velocities are high enough to produce turbulent wakes downstream of the mesh filaments, increasing their effective diameter and decreasing the fluid 'porosity' of the cage (Taylor 1948, Gerrard 1978).

An alternative explanation for the observed significant cage effect in the u_* = 2.0 cm s^{-1} treatment is that suspension of the larger-sized clams was more sensitive than that of the smaller ones to flow alterations caused by the cage. We do not think this is a valid explanation for several reasons. Firstly, there is no evidence for an increasing sensitivity of clam suspension to caging with increased clam size; instead, the small and large clams tended to have increased retention under the cage, whereas the medium size clams showed a mixed response. Secondly, because larger clams are capable of burrowing deeper than small ones, one would expect them to be less, and not more, sensitive to caging effects. Finally, we have no independent evidence for the "clam size" hypothesis, whereas we do have independent flow measurements in the cage that support the "flow threshold" hypotheses. For these reasons, we constrain our discussion to the latter.

Behavioral Effects on Clam Retention

A reduction in near-bottom flow velocity may enhance clam retention through both hydrodynamic and behavioral mechanisms. A critical shear stress is necessary to suspend clams of any particular size; reducing the shear stress below this level will result in more limited transport. Reducing the shear stress will also reduce the rate of sediment erosion, potentially allowing clams more time to burrow below the unstable sediment layer. Either or both of these processes are likely responsible for the relationship between higher shear stresses and enhanced clam transport reported in flume (Roegner et al. 1995, Dunn et al. 1999) and field (Emerson and Grant 1991) studies.

Although surficial sediment was eroded under the cage in our trials at u_* = 2.0 cm s^{-1}, the reduction in rate of erosion relative to uncaged flows may have allowed clams to remain buried in the sediment. In order to maintain access to the sediment-water interface, postlarval *M. arenaria* of 1.3 to 2.3 mm SL can only burrow as deep as their siphons are long, usually to depths of 7.5 mm or less (Zwarts and Wanink 1989). We speculate that *M. arenaria* may actively burrow to avoid being exposed and eroded as Sakurai and Seto (1998) demonstrated for the surf clam, *Pseudocardium sachalinensis* at the reduced shear velocities but not at ambient uncaged shear velocities. Thus we suggest that the reduction in shear stress offered by the cage at u_* = 2.0 cm s^{-1} sufficiently reduced the erosion rate so that 2.3 mm clams could maintain their position in the sediment and avoid resuspension.

Based on the siphon length-burial limitation, we expected to find that for any specific shear velocity, retention of larger clams would be greater than retention of the smaller clams. Although our data alone do not strongly support this hypothesis, comparison with another study demonstrates a retention-size relationship. We found that 25–35% of our 1.8 mm clams were retained at u_* = 1.8

TABLE 2.

Summary of ANOVAs examining the effects of cage and velocity on retention over a range of size categories.

Clam Size	Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Small	Cage	0.030	1	0.030	0.939	0.352
	Velocity	0.072	1	0.072	2.289	0.156
	Cage*Velocity	0.008	1	0.008	0.243	0.631
	Error	0.378	12	0.031		
Medium	Cage	0.005	1	0.005	0.609	0.457
	Velocity	0.016	1	0.016	1.912	0.204
	Cage*Velocity	0.001	1	0.001	0.104	0.755
	Error	0.066	8	0.008		
Medium*	Cage	0.015	1	0.015	2.099	0.185
	Velocity	0.448	1	0.448	64.70	<0.001
	Cage*Velocity	0.002	1	0.002	0.232	0.643
	Error	0.055	8	0.007		
Large	Cage	0.076	1	0.076	8.129	0.046
	Error	0.037	4	0.009		

* Separate ANOVAs were performed on two sets of mid-season clams because those tested at u_* = 1.7 and 1.8 cm s^{-1} were exposed to flow for only 10 min (Systat version 6.1).

cm s^{-1}, yet Roegner et al. (1995) reported that 0% of their 0.24–0.29 mm clams were retained at a comparable velocity ($u_* = 1.75$ cm s^{-1}). Thus smaller clams were more readily transported at this particular velocity and retention appeared to correlate with clam size.

Relevance to Field Applications

Our flume results suggest that mesh cages used in the field may enhance retention of recently settled bivalves in flow velocities above a critical level. The extent to which our flume experiments are applicable to field environments depends on how well they represent field situations hydrodynamically, biologically, and structurally. The shear stresses used in the flume experiments fall within those measured at Barnstable Harbor (authors' unpublished data), indicating that boundary-layer flows in the flume are a reasonable representation of field flows. The cultured clams burrowed actively, and their sizes covered the range observed in field populations during first season growth. The cage used in the flume had the same mesh size and composition (flexible mesh), but a much smaller overall size, as mesh enclosures used in the field. Thus, we expect our qualitative prediction (mesh enclosures enhance bivalve retention only above a threshold shear stress) to be broadly applicable, but the specific threshold level to depend somewhat on the geometry and mesh size of the cage.

A reduction in resuspension caused by caging will notably influence population density only if loss via resuspension is a significant source of total loss. Other processes influencing loss include mortality through predation, disease, and/or inadequate resources. Manipulative field experiments evaluating the relative influences of hydrodynamic resuspension and predation on *M. arenaria* recruits indicate that resuspension is a significant cause of loss, especially in smaller size-classes (authors' unpublished data). Therefore, we expect that the possible increase in retention caused by caging could have a significant impact on population density by the end of the first season.

Our flow and retention results are consistent with experimental field evidence suggesting that clam recruitment under cages could be enhanced at high flows. Marcotti and Leavitt (1997) tested the hypothesis that cages, in the form of suspended tents of plastic mesh (mesh size = 0.5 cm), would increase clam recruitment in field manipulations at Barnstable Harbor, Massachusetts. They found that the only site at which the cages significantly enhanced recruitment (Green Point) was one of the two sites with the highest tidal flow rates. Velocity records from the Green Point site indicated that over a 10 h period during a spring tide, 40% of the flow velocities corresponded to $u_* = 2.0$ cm s^{-1} or greater (authors' unpublished data). Thus velocities at Green Point commonly were high enough to produce the caging effect demonstrated in our flume experiments, suggesting that the enhanced recruitment may have been due to cage-induced retention.

Because mesh enclosures are more likely to retain clam juveniles in vigorous rather than quiescent flow environments, the hydrodynamic setting of a site is an important factor to be considered in their use. This consideration should be included with others, such as the potential of the enclosures to enhance initial settlement, enhance or deplete food resources or reduce mortality through predator exclusion, when predicting whether they are likely to increase population densities. Mesh enclosures are currently in use in multiple embayments along the New England coast and the results of these applications will help refine the cost-benefit analysis of their use and identify the habitats where they will substantially increase the numbers of harvestable soft-shell clams.

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GROWTH OF BUTTER CLAMS, *SAXIDOMUS GIGANTEUS* DESHAYES, ON SELECTED BEACHES IN THE STATE OF WASHINGTON

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ABSTRACT The butter clam, *Saxidomus giganteus* Deshayes, is a highly valued recreational shellfish species, but is currently of little commercial importance in Washington. Recently, a small-scale commercial butter clam harvest began, and interest in this species is expected to increase. Basic information on the biology and ecology of the clam is necessary to establish a sound management regime for recreational and potential commercial harvest of butter clams. Beaches studied were Birch Bay State Park, Double Bluffs, and Potlatch State Park. Growth rates were determined by measuring lengths-at-age for clams collected from each beach. Von Bertalanffy growth curves were produced using nonlinear regression analysis. Growth rates were significantly different among the three beaches, with the fastest growth occurring at Double Bluffs, followed by Birch Bay State Park, while clams at Potlatch State Park had the slowest growth.

KEY WORDS: *Saxidomus giganteus*, growth

INTRODUCTION

The purpose of this investigation was to assess whether growth of butter clams (*Saxidomus giganteus* Deshayes) on selected beaches in Washington differed. Beaches selected for this study were Birch Bay State Park, Double Bluffs, an Island County recreational area on the southwestern coast of Whidbey Island, Washington, and Potlatch State Park (Fig. 1). There was no evidence to suggest that growth rates might differ within the Puget Sound Basin, although different growth rates have been observed for butter clams from beaches within close proximity in British Columbia (Quayle and Bourne 1972).

The three beaches included in this study were surveyed for butter clams using methods similar to beach survey methods used by the Washington Department of Fish and Wildlife (WDFW). Clam shells were aged using the annular method, which appears to be the most reliable method of age determination for butter clams (Goong 1999). At least two researchers have independently verified deposition of annuli in the shell of the butter clam using mark and recapture studies in Washington and British Columbia (Houghton 1973, Neil Bourne pers. comm.). Growth rates were calculated using von Bertalanffy methodology (von Bertalanffy 1938).

MATERIALS AND METHODS

Study Areas and Habitat

Two hundred and forty-one butter clams were collected from three beaches for this study from August 17–19, 1997. Clams were collected from Birch Bay State Park on August 17, from Double Bluffs on August 18, and from Potlatch State Park on August 19.

Birch Bay State Park is located near Bellingham at the southern end of the Strait of Georgia. The beach has a gradual slope, and is characterized as primarily a sand-rock beach, with numerous larger cobblestones (up to 15 cm × 15 cm) on the surface. The beach has good road access, with parking less than 50 m from the water. This allows heavy recreational use, including clam harvesting, judging from the large number of clam harvesters observed and poor clam densities found on the beach. Other clam species observed on the beach included many Manila clams (*Tapes philippinarum*) and a few native littleneck clams (*Protothaca staminea*). Green shore crabs (*Hemigrapsus* sp.) were common. Water quality measurements, as recorded by the Washington Department of Ecology (WDOE) (Newton et al. 1997), showed surface temperatures averaged 7°C in winter and 10–13°C in summer. Salinity was fairly constant, ranging from 24–27 ppt throughout the year. The concentration of chlorophyll *a* was about 0.6 μg/L in winter, and about 3 μg/L during the rest of the year.

Double Bluffs Beach is located on the southwestern coast of Whidbey Island, in central Puget Sound. Substrate and topography of this beach was similar to that of Birch Bay. There was poor road access, with a parking area located approximately 1 km from the southern boundary of the clam bed. This beach probably has little recreational harvest, judging from the few clam harvesters observed, high clam densities, and large numbers of older clams found there. Other species of clams observed on this beach included small numbers of Manila clams, cockles (*Clinocardium nuttalli*), bent nose clams (*Macoma nasuta*), and horse clams (*Tresus capax*). There were also large numbers of green shore crabs and acorn barnacles (*Balanus* sp.). One juvenile cancrid crab was also observed. Sea surface temperatures averaged 8°C in winter and 10–12°C in summer, a temperature regime similar to that of Birch Bay. Salinity averaged 29 ppt throughout the year. Chlorophyll *a* concentrations were about 0.4 μg/L in winter, and about 4.3 μg/L in summer. These water quality measurements were similar to those found at the WDOE sampling station near Birch Bay. Lower salinity measurements for the station near Birch Bay may be due to the influence of freshwater from the Fraser River in southern British Columbia (Newton et al. 1997).

Potlatch State Park is at the southern end of Hood Canal near the southern end of the Puget Sound Basin. The topography of this beach was more complex than the other two beaches. Much of the beach was flat near the road, then sloped gradually upward toward the water, and then downward. However, some portions of the beach sloped gradually downward the entire distance to the water. Because of this, much of the flat area of the beach is flooded during high tide and had good clam populations, despite being fairly distant (50 m or more) from the water level during minus tides. Substrate of this beach was best characterized as mud-gravel, and there were no cobblestones on the surface. Sampling of this beach coincided with the final day of a three-day native tribal Manila clam harvest, during which butter clams were also collected. Thus, clam densities were poor, and most clams collected

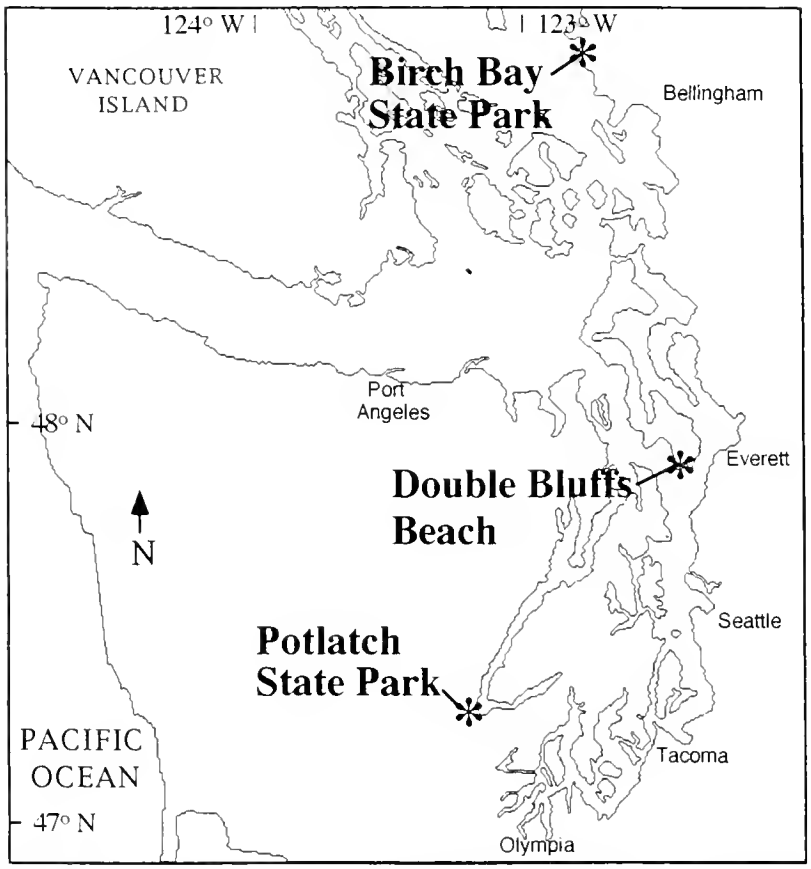

Figure 1. Map of the Greater Puget Sound Region showing locations of the three study sites.

were in the 3+ age class or younger. Other clam species observed on this beach included a few bent nose clams and numerous Manila clams. Surface temperatures averaged 7°C in winter and 14–18°C in summer. Salinity was highly variable, due to freshwater drainage from the Skokomish River and several small creek systems, but ranged from 16–26 ppt. Chlorophyll *a* concentrations were also highly variable, but tended to be quite low during summer months, averaging only about 0.9 μg/L. The two other beaches had steady concentrations of chlorophyll *a* from spring through autumn, with low chlorophyll *a* concentrations only in winter months.

Sampling Methods

Sampling was undertaken using survey methods similar to those employed by WDFW (Campbell 1996). A reference transect was established on each beach which ran perpendicular to the shoreline. The end of the transect nearest the high water line was situated approximately 30.5 m from the water line at the low tide mark. A 30.5 m length of rope was laid along the transect down to the water line. The rope was marked with a permanent marker every 1.5 m so it could be used to measure distances on the beach. Quadrats of 0.25 m^2 in area were established every 4.5–10.5 m along the length of the transect to ensure that clams were collected from different tidal heights. Positions of the quadrats occasionally had to be adjusted in order to account for beach topography. Subsequent transects were established 9–15 m from each other and the reference transect, and were also perpendicular to the shoreline. Quadrats were established along these transects in a similar manner and excavated to a depth of 0.5 m.

Quadrats were excavated using standard garden spades. As quadrats were excavated, rocks and other large debris were removed. Large butter clams were removed and collected. The remainder of the substrate was sifted through 1-cm mesh screens in order to collect smaller clams.

Analysis of Growth

Data were analyzed using the von Bertalanffy growth model (von Bertalanffy 1938):

$$l(t) = L_\infty - L_\infty e^{-k(t - t_0)},$$

- $l(t)$: length at age t
- e: base of the natural logarithm
- t_0: time when length is theoretically zero
- k: von Bertalanffy growth constant
- L_∞: asymptotic maximum length of the clam.

Model parameters were estimated using nonlinear regression analysis, as suggested by Gallucci and Quinn (1979), in lieu of the Ford-Walford method. Gallucci and Quinn (1979) noted that use of nonlinear regression facilitates quantitative comparisons among the parameters of the equation. Commonly, comparisons are made on the growth constant (k) only. Since it is standard practice to report differences in k as representing differences in growth, this convention was followed in the present study. An F test was utilized in order to make statistical comparisons among parameters

(Neter et al. 1996). Statistical analyses were performed with SPSS 6.1.1S for the Power Macintosh.

RESULTS

Von Bertalanffy Growth Analysis

Von Bertalanffy growth models resulting from these parameters are plotted in Figure 2. Portions of these curves corresponding to the first 9.5 years of growth are redrawn in Figure 3 to exhibit greater detail.

Figure 3 clearly shows that growth is different among beaches. Fastest growth occurred at Double Bluffs, followed by Birch Bay, while slowest growth was observed at Potlatch. However, although the rate of growth was slower at Potlatch, it did not appear to decrease over time as quickly as it did at the other beaches. This is probably due to an inadequate number of older specimens in the sample from Potlatch. Most clams collected from Potlatch were in the 3.5-year age class or younger. The result is a paucity of growth data beyond the first four years, possibly leading to a poorly fitting growth model. One indicator of this possibility is the L_∞ calculated for Potlatch shown in Table 1. This is an unreasonably large value for maximum clam length. However, the early part of the curve fits the data. Thus, the model may still adequately describe growth for the first several years.

The growth constants (k) listed in Table 1 support conclusions drawn from Figure 3. Fastest growth occurred at Double Bluffs, while slowest growth occurred at Potlatch. Differences among the three beaches were significant at the $\alpha = 0.05$ level.

DISCUSSION

Differenees Among the Three Beaches

Of the three beaches, Potlatch is the most diverse. Potlatch is a mud-gravel beach, which is somewhat different from the preferred butter clam substrate of sand, shell, and gravel (Quayle and Bourne 1972). The substrate at Potlatch is more suited to Manila clams (*T. philippinarum*), as evidenced by the numbers of Manila clams observed on this beach. Double Bluffs, where the fastest growth occurred, and Birch Bay are both sand-rock or sand-rock-shell beaches. In addition, butter clams were most abundant at Double Bluffs.

Potlatch is more influenced by freshwater drainage than the other two beaches. Results of the present study indicate that butter

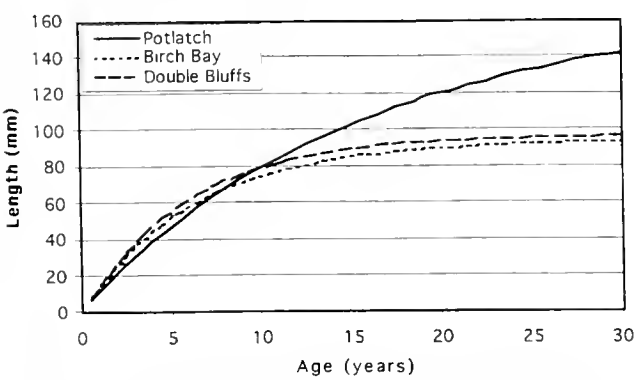

Figure 2. Von Bertalanffy growth curves for butter clams from Potlatch State Park, Birch Bay State Park, and Double Bluffs Beach, Washington.

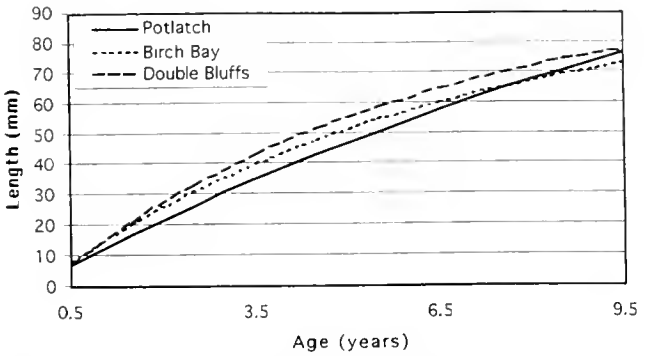

Figure 3. Von Bertalanffy curves for butter clams from Potlatch State Park, Birch Bay State Park, and Double Bluffs Beach, Washington, plotted to 9.5 years.

clam growth may be positively correlated with salinity. Although no other butter clam studies support this suggestion, studies on oysters suggest that oyster growth may be influenced by differences in salinity (Toro et al. 1995, Mallonee 1989). Robert et al. (1993) conducted a study of Manila clam growth at three sites, one of which was oceanic and the others were estuarine. Clam growth was fastest at the oceanic station, and they speculated that differences in growth were due in part to differences in salinity. Like butter clams, Manila clams are venerid clams. Bardach et al. (1972) reported that the optimum salinity range for Manila clams was about 24–32 ppt, which is the normal salinity range for Puget Sound. This salinity range may also be optimal for butter clams. Average salinities recorded at Potlatch are normally below this range, ranging from 16–26 ppt (Newton et al. 1997).

As noted in Study Areas and Habitat, surface water temperatures at Potlatch tended to have greater fluctuations than temperatures at the other beaches. This may be important if butter clams are better adapted to more stable growing areas. Further, water temperatures in summer at Potlatch can be particularly high relative to those at the other beaches. Irregularity in year round water temperatures and excessive summer temperatures could cause a sufficient amount of stress to have a negative impact on growth. Temperature has been shown to be an important determinant of bivalve growth in several species, including another venerid clam, the northern quahog (*Mercenaria mercenaria*), scallops, and oysters (Crockett 1988, Claereboudt 1994, Toro et al. 1995). Mann and Glomb (1978) and Mann (1979) showed a negative correlation between temperature and growth in Manila clams, with fastest growth occurring at 12°C. Growth was also correlated with water

TABLE 1.

Parameters of the von Bertalanffy growth equation for butter clams from three beaches in Washington, calculated using all annulus lengths.

Beach	k ± SE	L_∞ ± SE	t_0 + SE
Potlatch State Park	0.064 ± 0.010	165.903 ± 20.243	−0.222 ± 0.048
Birch Bay State Park	0.157 ± 0.010	93.870 ± 3.218	−0.083 ± 0.051
Double Bluffs Beach	0.172 ± 0.006	96.905 ± 1.542	0.043 ± 0.032
Total	0.140 ± 0.004	105.157 ± 1.892	−0.004 ± 0.026

temperature in Bourne (1982), but the correlation was positive. Fastest growth in natural populations of Manila clams occurred in the Strait of Georgia, where surface water temperatures are highest, commonly above 15°C in summer (Hollister and Sandes 1972). However, Mann's results were from laboratory experiments, and some other factor may be responsible for the observed differences in growth in British Columbia.

In addition, food availability was more irregular at Potlatch, as indicated by chlorophyll *a* concentrations. There appears to be higher food availability during spring and autumn. Winters at Potlatch are times of low food availability. Unlike the other beaches, there was also low food availability at Potlatch during summer. At Double Bluffs and Birch Bay, food availability was consistent from spring through autumn. This is supported by the dissolved inorganic nitrogen levels at Potlatch, which are frequently undetectable from May through August in the surface water (Newton et al. 1997). Dissolved inorganic nitrogen is generally considered the limiting nutrient in marine ecosystems (Valiela 1984). Thus, maximum primary productivity at Potlatch may be a growth limitation for bivalves at Potlatch. Food availability has been shown to be among the most important determinants of bivalve growth, including growth in *M. mercenaria* (Claereboudt 1994, Mallonee 1989, Crockett 1988). Foe and Knight (1985) found that Asiatic clams (*Corbicula fluminea*) are probably food limited in the Sacramento-San Joaquin Delta, even with chlorophyll *a* concentrations as high as 62.5 µg/L, which is an order of magnitude higher than concentrations found in Puget Sound.

Finally, there is also a gradient of human activity at these three beaches. Potlatch may be a site of intense harvesting, if not for great numbers of butter clams, then certainly for Manila clams. Also as noted earlier, there was a commercial harvest by Native American harvesters. Birch Bay is not subject to commercial harvest, but is heavily used for recreational purposes, since the roadway allows direct road access to the beach. Double Bluffs is also not available to commercial harvesting and probably has little recreational use of the butter clam resource due to distance of the clam bed from the road access. One effect of heavy use may be substrate compaction. Studies conducted in Washington demonstrated that substrate compaction may be an important cause of low productivity on clam beaches (Toba et al. 1992). Increased human activity in an area has been shown to decrease biological integrity, resulting in decreased species diversity, and may have negative impacts on growth and health in general on all taxa in a given ecosystem (Karr 1981). Houghton (1973) showed a high correlation between taxa richness and growing areas with high clam densities, strong growth, and high productivity. Thus, bivalve growth may be negatively correlated with a gradient of human activity. Indeed, this may be an important factor in growth on popular recreational clam beaches, although it is probably negligible on more remote beaches, such as Double Bluffs.

Patchiness in age distribution may have affected growth analysis for Potlatch State Park. In the von Bertalanffy analysis for Potlatch, the lack of strong representation of older clams likely contributed to the computation of an unrealistic L_∞, causing the Potlatch curve to eventually cross the others. If the true asymptote for Potlatch is closer to those of the other beaches as is suspected, the curve would probably not cross the others. It should be noted that the calculated parameters are still adequate to describe the observed growth for at least the first four years. Due to poor data for subsequent years, the model does not provide an adequate description of growth for later years.

CONCLUSIONS

1. Butter clams from different beaches in Puget Sound, Washington have different rates of growth. Fastest growth occurred at Double Bluffs, and slowest growth occurred at Potlatch.
2. The legal size limit for butter clams in Washington probably should be increased. The current size limit for butter clams is 38 mm. According to results of growth analysis, clams in this size class would be only about 2–3 years old. It is suggested that the size limit be increased to about 63 mm, the size limit in British Columbia. Clams in this size class would be about six years old, and would have had opportunities to spawn.
3. Since rates of growth are different in different growing areas, it is suggested that a more localized management approach for the resource be utilized in order to protect the productivity of the stock. A generalized management strategy will not optimally manage the resource. This would require growth analyses to be performed in any area that has any commercial or intense recreational harvests for butter clams.

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REGENERATION OF THE INHALANT SIPHON OF *DONAX HANLEYANUS* (PHILIPPI, 1847) (BIVALVIA, DONACIDAE) FROM ARGENTINA

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ABSTRACT *Donax hanleyanus* (Philippi, 1847) is the southernmost *Donax* species in the American Atlantic. It inhabits intertidal fine grain sandy shores in northern Buenos Aires Province. As with other species in this genus, these animals survive under pressure of "cropping" by fish that feed on their siphons. The inhalant siphon is a complex organ; its tip contains a system of branched tentacles that permit particle selection of medium and large grains and prevent their entry to the pallial cavity of the animal. To estimate the regeneration speed of the amputated siphon under laboratory conditions, a study of the regrowth sequence after an artificial cut was performed at regular intervals for complete 10-day periods. The observations *in vivo* under the microscope were correlated with those made by histology at the same time intervals. Results indicate that at 24 h after amputation, rudiments of the primary tentacles are observed, and the siphon is fully active in selecting particles at the fifth day (i.e., the process of regeneration of the primary, secondary, and tertiary tentacles is completed within ~5 days). After this, a period of growing and tentacle ramification follows, even though the result is a siphon with tentacles less ramified than the original.

KEY WORDS: bivalve, inhalant siphon, siphonal regeneration, *Donax hanleyanus*

INTRODUCTION

Beach clams of the Tellinacean genus Donax inhabit intertidal sandy beaches in most parts of the world. *Donax hanleyanus* (Philippi, 1847) is the only Donax species occurring throughout the Argentine littoral. These bivalves are constantly flushed out of the sand by the pounding surf; they are adapted to live on exposed shores, and they do not occur on protected beaches or in shallow bays. *D. hanleyanus* require an environment in which there is a fair amount of wave action to keep the sand aerated and clean, to keep organic detritus in suspension, and to allow tidal migration (Ansell 1983, Ansell & Trevallion 1969, Mori 1938, Mori 1950, Narchi 1978, Penchaszadeh & Olivier 1975).

The siphons of *D. hanleyanus* are very flexible and extensible, as are those of other tellinacean bivalves. When buried, *D. hanleyanus* extends its inhalant siphon through the sediment into the water above (~1.5 cm for a 2-cm animal). Juvenile flatfish (Trevallion 1971, Gillbert & Suchow 1977, de Vlas 1979), crabs (Hughes 1969), and birds (Ansell 1981) feed on tellinacean siphons. Some studies have included an examination of the process of wound healing or the physiological basis of the regeneration, focusing on the amount of regenerated tissue (Hodgson 1982a, Hodgson 1982b, Peterson & Quammen 1982).

Siphon regeneration after amputation has been described for the tellinaceans *Tellina tenuis* da Costa (Trevallion 1971), *Scrobicularia plana* (da Costa), *D. serra* Röding (Hodgson 1982a, Hodgson 1982b), and *D. vittatus* (Ansell et al. 1999).

The external surface of the tip of the inhalant siphon of *D. hanleyanus* has six branched primary tentacles, six branched secondary tentacles between two primary tentacles, twelve small tertiary tentacles between a primary and a secondary tentacle, and 24 smaller quaternary tentacles between two tertiary tentacles (Fig. 1), which are very mobile. These tentacles form an intricate sieving device (Narchi 1978).

The siphons of *D. hanleyanus* are very sensitive to vibrations. They are richly innervated and rich in sense organs, at least near the tips (Hodgson 1982b, Ansell et al. 1999). It would be beneficial to the animal if damaged or lost parts of the siphons could regenerate rapidly after injury so as to regain normal function.

The aim of this study was to examine how soon the normal tip of the inhalant siphon of *D. hanleyanus* is regenerated, focusing on the proper function of filtration, and to study the morphological characteristics of the siphon regeneration by simulating the effect of a cropping predator.

MATERIALS AND METHODS

The animals were collected from sandy beaches in Punta Mogotes (Mar del Plata), at low tide. We selected specimens that were approximately 2 cm in shell length. Previously in the lab, aquaria had been conditioned with $CaCO_3$-saturated seawater at 35‰ salinity and pH 7.8. The experimental aquarium device consisted of two fishbowls, one of 200 l and the other of 10 l. The small one was inside the big one. Sand extracted from the sampling areas was placed inside the small one. Water was pumped from the big one

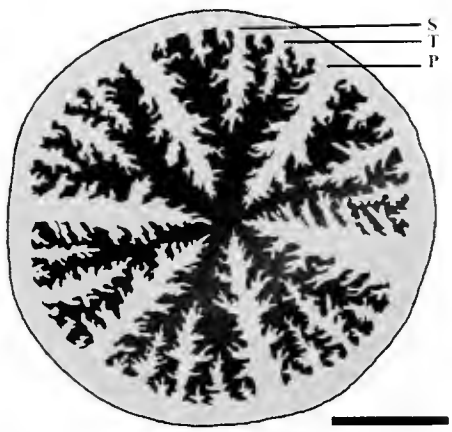

Figure 1. View of the inhalant siphon tip of *Donax hanleyanus* showing tentacles and ramifications. P: primary tentacles; S: secondary tentacles; T: tertiary tentacles. Scale bar: 0.5 mm. (After Wade, 1969)

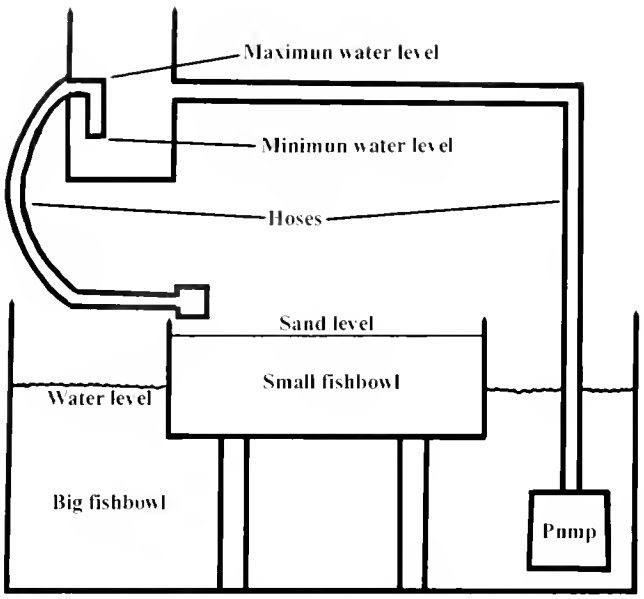

Figure 2. Aquaria device scheme to recreate the high-energy conditions of the natural environment of *Donax hanleyanus*.

to a third bowl located 1 m above. Water from this third bowl siphoned its contents into the small fishbowl and in this way it kept the sand and water aerated and turbulent, simulating the high-energy conditions of the natural environment of these animals (Fig. 2). The animals were exposed for at least 10 days to this aquarium system, and the temperature was kept between 20 and 22°C during the experiments.

After the adaptation period, 30 *D. hanleyanus* were placed in a shallow glass dish containing sea water and left undisturbed until the siphons were extended. The tip of the inhalant siphon was removed from un-narcotized individuals using ophthalmic scissors (1 to 2 mm from the tentacle crown). Five siphons, used as controls of the initial regeneration point, were fixed immediately in Bouin solution for 24 h. The remaining 25 animals were left to recover in the running sea water system described above, which contained a layer of sand deep enough to allow the animals to burrow normally. These animals were sacrificed in groups of five at 1, 3, 5, 7, and 10 days, always extracting the siphon tip and fixing it in Bouin solution. All of these fixed siphon tips were processed histologically. A dehydration and paraffin inclusion protocol was followed, and transverse sections were cut at 7 microns, after which hematoxylin-eosin tint was used.

Because it is difficult to obtain histological cuts of the siphon tip always in the correct orientation, we chose the best ones for analysis and photography.

The changes that followed the initial cut on the living animals were observed daily under a stereoscopic microscope to give *in vivo* corroboration.

Drawings were made from observations under the stereoscopic microscope for living animals and the histology microscope for fixed siphons. Each drawing is a composit of all the information of a specific regeneration point. The qualitative regeneration curve resulted from digital images of these drawings at 600 dpi in black and white (siphon black and background white). Using a computer, we counted the number of pixels in black to calculate a percentage index of coverage as (number of black pixels)/(number of total pixels) × 100. This index was graphed against time in days.

This protocol was repeated twice to provide experimental corroboration.

All the experiments were made with the animals in starving conditions, and a control group of undisturbed non-excised animals was used to test viability during the experiments.

RESULTS

For the histological interpretation of the complex tentacle system of the inhalant siphon of *D. hanleyanus*, we classified the different tentacles according to the shape and quantity of the branches. This primary filtration organ comprises 6 branched primary tentacles, 6 branched secondary tentacles between the primary tentacles, and 12 tertiary tentacles between a primary and a secondary tentacle. Every tentacle thinner than a tertiary tentacle was considered as a branch.

The observations made under stereoscopic microscope revealed that the tentacles not only form a net that selects the grain size but also that the tentacles have the capacity to change the diameter of the siphon opening. This is shown in Figure 3, which illustrates how the siphon acts to occlude the opening and prevent the entry of large particles of sand. The structure of each tentacle and of the whole siphon, comprised of longitudinal and circular muscles, enables it to contract and elongate (Fig. 4).

The regeneration sequence of the inhalant siphon is shown in Figures 5 and 6. The first rudiment of the new six primary tentacles is visible 24 h after the cut, and they are developed in 48 h. After the third day the secondary tentacles appear, and after the fifth day they are well differentiated from each other. On the seventh day all tentacles (i.e., primary, secondary, and tertiary) are very well developed, and at this time we can affirm that the siphon is totally functional. In the later stages its branches began to be observed in the tentacles, but from this time the regeneration rate diminishes

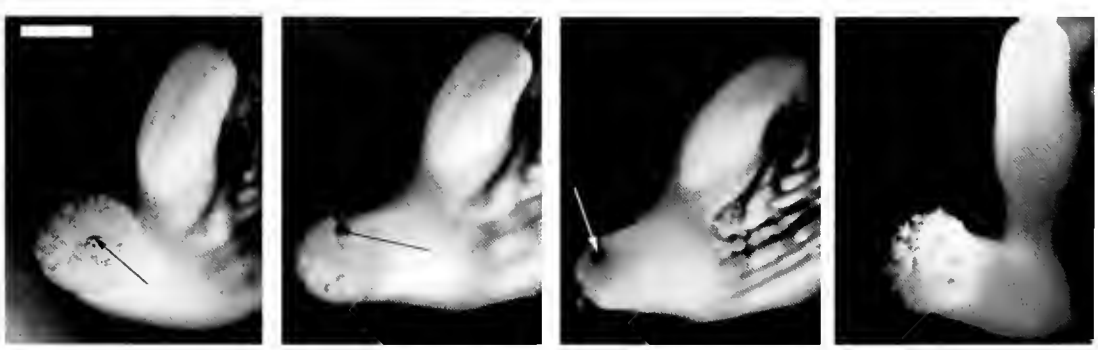

Figure 3. Sequence of rejection of a sand particle (arrows) by an intact inhalant siphon of *Donax hanleyanus*. Scale bar: 4 mm.

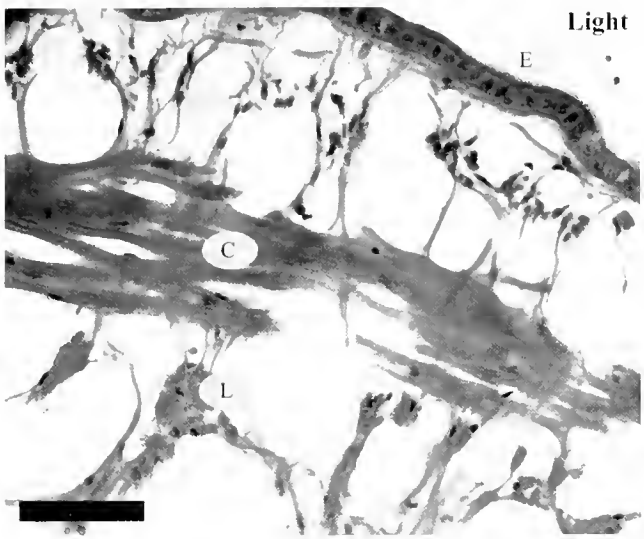

Figure 4. Inhalant siphon transverse cut (7 microns) showing muscle structure. C: circular muscles; L: longitudinal muscles; E: cubic ciliated epithelium from the internal siphon surface. Scale bar: 50 microns.

notably. Moreover, after 10 days the complexity and ramification quantity is still not the same as the control siphon (Fig. 5).

An approximation of the regeneration rate is shown in Figure 6, where the degree of regeneration point corresponds to standardized drawings. In the figure a lag period in the regeneration can be observed during the first 24 h. This is followed by a period of very intensive regeneration between the second and fifth day, and finally the process is decelerated until the end of the regeneration.

The animals used as controls (undisturbed animals) remained alive after 40 days, and no mortality was detected during the experiments in the excised animals. We did not record any difference on the siphon-restoring rate during the second experiment series.

DISCUSSION

The morphology and internal anatomy of the siphons of Tellinacea have been well described (Yonge 1949), although in *Donax* there are generally more tentacles than Yonge supposed, and in life most extend across the siphonal aperture forming a protective screen that excludes sand particles rather than all being turned back, as Yonge described. The siphons of *D. hanleyanus* do not differ significantly from those of the closely related European species *D. trunculus* L. (Moueza & Frenkiel 1974) and *D. vittatus* (Ansell et al. 1999). The siphons of these three species are closely similar in many respects to other Donax species (Duval 1962,

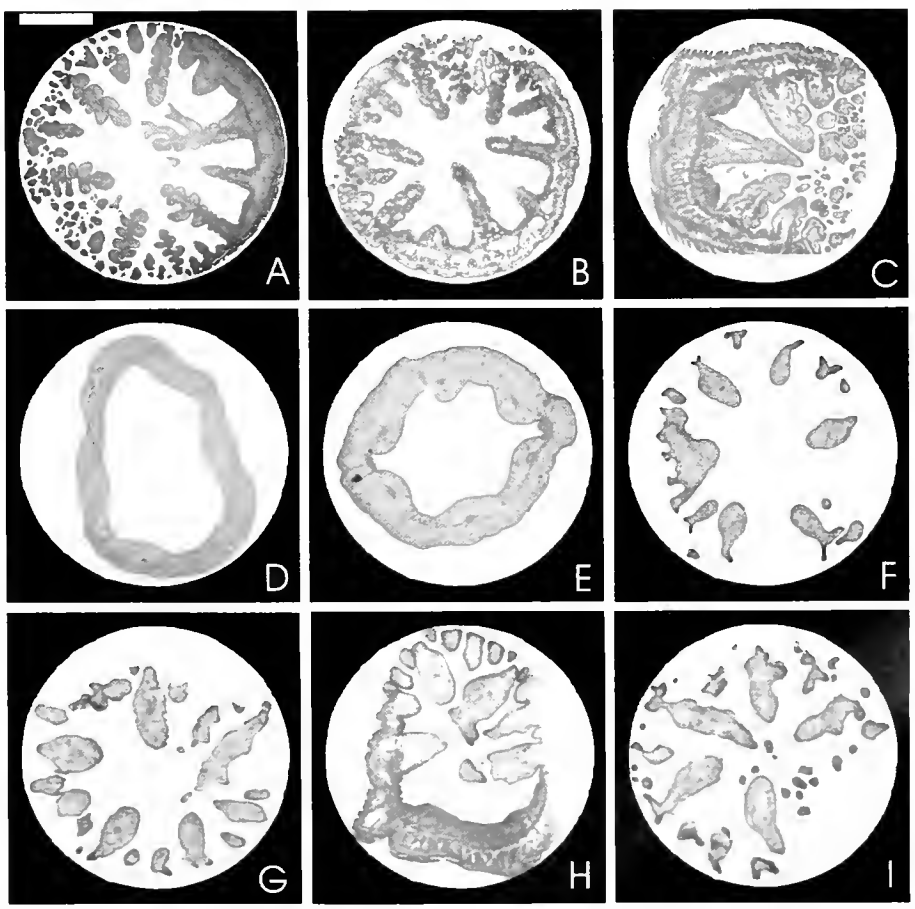

Figure 5. Regeneration of the inhalant siphon tip of *Donax hanleyanus*. A, B, and C: Intact siphon. D: Siphon wall immediately after amputation. E, F, G, H, and I at 1, 3, 5, 7, and 10 days after amputation, respectively. Scale bar: 1 mm.

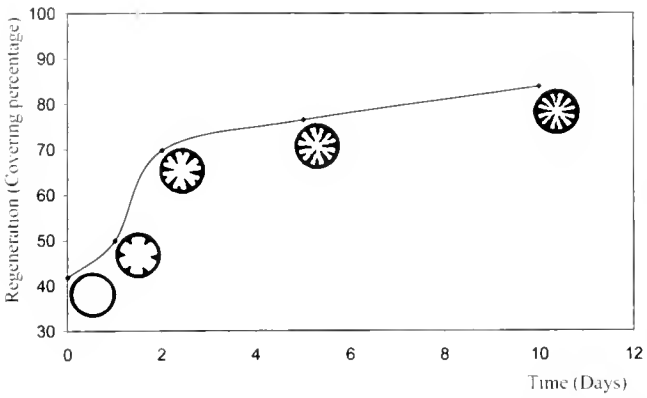

Figure 6. Rate of regeneration of the tentacle structure of the inhalant siphon of *Donax hanleyanus*. Each point is in correspondence with standardized drawings.

Wade 1969, Ansell 1983a, Ansell 1983b), although there is variation among members of the genus in the numbers of tentacles comprising each series (reflecting the number of siphonal nerves) and considerable variation in the complexity of their branching structure, especially around the tip of the inhalant siphon.

The study of siphon regeneration in bivalves is relevant to the impact of predation, mainly by fish and crabs, and the adaptations that bivalves require to survive to this impact (Edwards et al. 1970, Trevallion 1971, Lockwood 1980, Hodgson 1982a, Hodgson 1982b, Petersen & Quammen 1982, Pekkarinen 1984, de Vlas 1985, Ansell & Gibson 1990, Coen & Heck 1991, Bonsdorff et al. 1995, Ansell et al. 1999). The cropping of the siphons without killing the prey represents a way of conserving prey resources (i.e., it relaxes the predation pressure because it does not cause mortality) (Ansell 1983a).

The regeneration of the inhalant siphon in *D. hanleyanus* can be considered fully completed, in terms of siphonal function, between the fifth and the seventh day of regeneration. After this period the siphon continues growing in complexity and perhaps also in length, even beyond the tenth day. Hodgson (1982b) found that regenerating primary siphonal tentacles of *D. serra* could be detected as short lobes by 72 h following amputation. Within a further 24 h, these tentacles had elongated; by day 4 they had developed into tentacles, and secondary tentacles were also growing; by day 5, primary and secondary tentacles were large and multilobed, and tertiary tentacles were appearing; and by 7 to 8 days after amputation, the tentacles had attained their former size and appearance. Ansell et al. (1999) found very similar results for *D. vittatus*. *Tellina tenuis* da Costa, *Scrobicularia plana*, and *Macoma balthica* all lack elaborate siphonal tentacles, having only simple siphonal lobes. Hodgson (1982a) reports that these are fully reformed by 7 days after amputation in *S. plana*. In *M. balthica*, traces of the lobes are seen 2 days after amputation, and the lobes are fully formed again after 7 days (Pekkarinen 1984). The time course found here for the differentiation of the siphonal tentacles of *D. hanleyanus* was similar to that of *D. serra* and *D. vittatus*.

However, most of these studies concentrate on the later siphon regeneration, quantifying the regenerated tissue after months of amputation, and found that the siphon continues to grow at a rate that depends on the quantity of removed tissue at the beginning of the experiments. In *T. tenuis*, *S. plana*, and *D. serra*, regrowth proceeds at approximately 17–25% of the (undamaged) siphon weight per week (Hodgson 1982a), whereas the observations of de Vlas (1985) indicate a somewhat lower rate of regrowth in *M. balthica* of approximately 10% per week, with the regrowth rate proportional to the amount lost. Pekkarinen (1984) also found a relatively slow rate of regrowth for *M. balthica*; when one third of the siphon was removed, it took 3 mo to regrow to its normal size. The rate of regrowth was not measured for *D. hanleyanus* in this study but may be expected to be similar.

These authors also found that a lag period followed the first 24 h after the cut and point out that in this period there is a reorganization of connective tissue in the wound area. This is also seen in *D. hanleyanus* during the first 24 h after the cut; that is, the amputated siphon does not undergo any remarkable morphological change in this first period after amputation.

The evidence obtained from this study, together with the results of Hodgson (1982a, 1982b) Pekkarinen (1984), and Ansell et al. (1999), confirm that inhalant siphon regeneration is a universal process inherent to species belonging to the Tellinacea superfamily. Moreover, all the species studied up to now suggest that the regeneration rate is very similar regardless of latitude, temperature, and intensity of wave action.

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SHELL GROWTH AND BIOMETRY OF THE STRIPED VENUS *CHAMELEA GALLINA* (L) IN THE MARMARA SEA, TURKEY

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ABSTRACT Growth of the striped venus *Chamelea gallina* (L. 1758) in the northern Marmara Sea was studied by aging the shells. Internal growth bands with a seasonal pattern were identified on cross sections of the shell. During the period of reduced growth in October and February, a narrow hyaline band is formed. A large opaque band is formed between March and October. In the longest period of maximal growth, the increment coincides with the summer thermal maxima. *C. gallina* grows rapidly, reaching a length of 15 mm at the end of the first year and 21 mm at the end of the second year. At the end of the fourth and the fifth year, the length reaches 26 and 29 mm, respectively. The study of functional regressions permits us to conclude that the growth is allometric.

KEY WORDS: bivalve, *Chamelea gallina*, biometry, age, growth, Marmara Sea

INTRODUCTION

Growth rings on shell surfaces have been widely used to make inferences about age and growth rate in bivalves. However, in some species it is difficult to recognize real seasonal growth rings from false rings that may form under different stress conditions (e.g., storms, extreme temperatures, diseases, spawning, gonad development, dredging, etc.). (Polenta 1993, Deval 1995, Gaspar et al. 1995). Moreover, in older specimens the growth marks are so close to each other and to the shell margin that it may be difficult to identify them correctly.

Calcium carbonates and organic matter are deposited in the shell layers with a seasonal pattern (Panella & Mac Clintock 1968, Rhoads & Lutz 1980). In cross sections, these shell layers may be recognized by their optical characteristics and therefore are used to age bivalves.

Cross-sectioning has already been used successfully to age venerid clams (Richardson 1988, Ramon & Richardson 1992, Polenta 1993, Arneri et al. 1995). This method has also been used in the present investigation to study the growth of *Chamelea gallina* in the Marmara Sea. The most commonly used method to determine the rate of bivalve growth is to measure the changes in the dimensions of the shell. In the case of the striped venus *C. gallina*, the shell length along the anterior–posterior axis and the shell height measured from the umbo to the shell margin have been used.

C. gallina is found in throughout the Mediterranean Sea, including the Black Sea. Along the Atlantic coast of northern Europe it is replaced by the closely related species *C. striatula* (Backeljau et al. 1994). Both species are preferential, distributed on coastal fine sands (Guillou & Sariau 1985). *C. gallina* can be present in high densities in the Marmara Sea and in the Black Sea (Deval & Oray 1992, Deval 1995). The exploitation of *C. gallina* in Turkish waters started 14 y ago, and the yearly catch had reached 41 thousand tons by 1994. The striped venus plays an important role in the economy of Turkey, and this species is totally exported.

MATERIALS AND METHODS

Between February 1993 and April 1994 at approximately monthly intervals, samples of *C. gallina* were collected by dredging (Picard 1965) in depths of 4–7 m in a site off Tekirdag (Barbaros), in the northern Marmara Sea (Fig. 1). During the research period, 2,887 specimens were collected. Measurements of the length, height, width, total weight, shell weight, and meat weight were made with a vernier caliper, which was accurate to 0.1 mm, and with a digital balance, which was accurate to 0.01 g. The minimum and maximum values of the arithmetic mean, variance, coefficient of variance, and standard error (SE) were calculated, and the results were discussed (Valli et al. 1977, Valli et al. 1985, Valli et al. 1986). Log y = Log a + b. Log x logarithmic model, which could easily be turned into $y = a \cdot x^b$, was used for biometric changes of *C. gallina* (Ricker 1973, Ricker 1975).

Generally, for each 1-mm length class, five representative shells were sectioned. The right valve of the shell was cross-sectioned from the umbo to the ventral margin along the line of maximum growth, mounted on a glass slide, and then ground and polished on a lapidary wheel to obtain a section of approximately 20–30 μm. The section was then examined using a dissecting microscope under reflected light at low magnification. A total of 403 sections were prepared in this way and could be used for the interpretation of distinct annual increments. Shell sections were measured with video-analysis software, processing on a personal computer where the images were recorded through a video camera connected to the dissecting microscope. A complete record of size was obtained for each clam by measuring distance (height) from the umbo to the ventral margin of each hyaline band. Shell length was then transformed in shell height using the following regression:

$$\text{Length} = 0.491 + 1.062\ \text{Height}\ (r^2 = 0.996;\ n = 536)$$

In the Marmara Sea, *C. gallina* has a rather long spawning season, with a peak in June-July (Deval 1991). Therefore, to compute the parameters of the Von Bertalanffy growth equation June 1 was chosen as conventional birthday for all individuals considered in the length-at-age key:

$$L_t = L_\infty[1 - e^{-K(t-t_0)}]$$

where K is the Von Bertalanffy growth constant, L_∞ is the asymptotic maximum length, and t_0 is the age at time zero.

RESULTS

During the study, the minimum measured length and the maximum measured length were 3.6 mm and 34.5 mm, respectively. The mean length was found to be 20.1 ± 0.12 mm (±SE). The most common repeated values of length was 20 mm (by 6.8%), 19 mm,

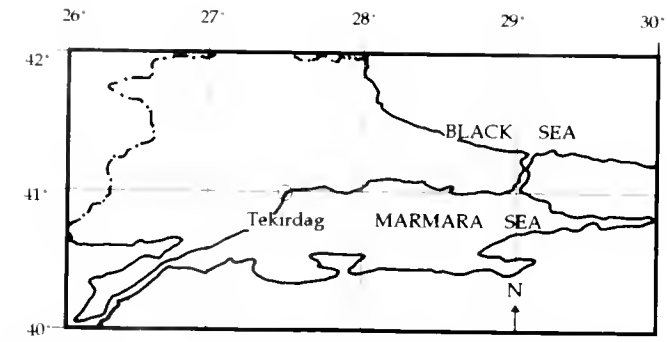

Figure 1. Location in the Marmara Sea where *Chamelea gallina* samples were collected.

and 21 mm (both by 6.6%). The length frequency distribution of *C. gallina* samples is shown in Figure 2.

The regression relationship for length and height with weight for *C. gallina* was positively linear. However, the relationship of the dimensional and the weight parameters (length/weight) was exponential. As seen in Table 1, when the regression coefficient between the two length variables (or the two weight variables) is $b \neq 1$, there is allometry in growth; when $b = 1$, there is isometry in growth. Likewise, when $b = 3$ between the length and growth variables there is isometry; when $b \neq 3$, there is allometry. Accordingly, between all variables there is an allometric growth.

Aging was carried out on 403 cross sections by two independent readers. Agreement was achieved on 297 shells (73.7%); the other shells were not further considered. To determine the periodicity in the formation of the hyaline band, the distance from the shell ventral margin to the nearest hyaline band was measured in all clams in the size range of 11–25 mm. When this distance did not exceed 1 mm, the hyaline band was considered "at the margin band" (Fig. 3). Of the 297 shells with hyaline bands, 198 shells had margin bands. The seasonal distribution of 198 *C. gallina* with bands at the margin of 11–25 mm is given in Table 2.

As seen Table 2, even though the building of the hyaline bands during the year is seen, the percentage of shells building hyaline bands in the warm summer season is very low (5.3%). However, the formation of hyaline bands appears to increase in the winter.

C. gallina in the Marmara Sea spawns from May to September, with a peak in early summer (Deval 1991). Therefore, individuals in the first winter of life exhibit quite different sizes. Specimens born earlier (in May) benefit from the appropriate conditions for rapid growth during the summer. By winter they may already have attained a length of 18–19 mm. However, specimens born at the end of the spawning season (August-September) benefit from the optimal environmental conditions. For a short period and in winter

TABLE 1.

Regression and confidence intervals of allometric growth for biometrics parameters of *Chamelea gallina*.

Variables (Dependent-Independent)	*n*	R	Log a	b	95% Confidence Intervals
H-L	536	0.998	−0.069	1.024	0.9373–1.1107
W-L	232	0.984	−0.303	1.005	0.9895–1.0206
SW-L	322	0.992	−3.544	2.900	2.8598–2.9393
MW-L	42	0.720	−4.771	3.265	2.6229–4.2676
TW-L	536	0.993	−3.383	2.902	2.8728–2.9314
SW-TW	367	0.995	−0.137	0.976	0.9666–0.9858
MW-TW	42	0.807	−1.039	1.180	0.9056–1.4547

H = height; L = length; W = width; SW = shell weight; MW = meat weight; TW = total weight.

they may be only 2–3 mm in length. Usually in the following summer these specimens have an above-average growth rate. The growth rate has a high interindividual variability. A specimen of 19 mm in length may be 1–3 y old (Fig. 4; Table 3).

The routine included in the software package FISAT (Gayanilo et al. 1996) was used to estimate the parameter of the Von Bertalanffy growth equation, together with their asymptotic standard error.

$$L_\infty = 33.46 \pm 1.32 \text{ mm} \qquad K = 0.37 \pm 0.067 \qquad t_0 = -0.69 \pm 0.30 \text{ y}$$

The theoretical mean length at age and the annual growth computed according to the above parameters of the Von Bertalanffy growth equation are listed in Table 4.

In the northern Marmara Sea, *C. gallina* has a rather rapid growth during the first 2 y of life (Table 4) and reaches the minimum marketable length of 23 mm in less than 3 y. The live-weight annual increment is highest during the third year of life (Fig. 5). Growth rates, both in length and weight, are much lower in older individuals, and the maximum size attained in the Marmara Sea is significantly lower than in the Adriatic Sea. The mean life span in the *C. gallina* population of the northern Marmara Sea seems not to exceed 5 or 6 y (shell length 29–30 mm). Only 2.2% of the sampled specimens were larger than 30 mm, indicating a rather high exploitation level in the investigated area.

DISCUSSION

This research is the first analysis on the growth and age of *C. gallina* in the Marmara Sea, giving the most detailed biometric parameters of *C. gallina* in Turkey.

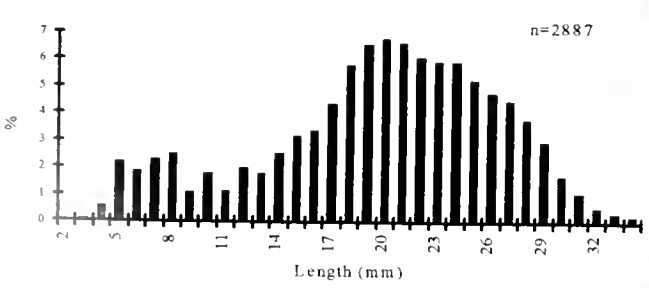

Figure 2. The length–frequency distribution of *Chamelea gallina* samples.

Figure 3. "At the margine band" on the coss-section of the shell of *Chamelea gallina*.

TABLE 2.

Position of the hyaline band nearest to the ventral margin observed in samples caught in various seasons.

Date	Sections (*n*)	Shells with Hyaline Band "at the Margin" *n* (%)
June 23, 1993	75	4 (5.3)
October 4, 1993	44	8 (18.2)
February 6, 1994	79	44 (55.7)

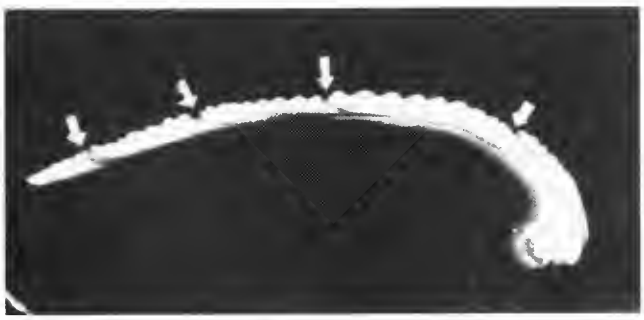

Figure 4. Thin section of *Chamelea gallina* of the Northern Marmara with arrows showing the annual growth (hyalin band) increments (length 27.9 mm, age 4+; data from the October sample).

TABLE 3.

The lengths of *Chamelea gallina* in winter the formation of the hyaline growth bands.

Number of Annual Hyaline Bands	Records (*n*)	Minimum Length (mm)	Maximum Length (mm)	Average Length*
1	300	2.3	19.3	8.79 ± 4.258
2	252	12.6	25.6	18.73 ± 2.530
3	161	19.1	29.7	23.58 ± 2.117
4	92	22.0	32.0	26.37 ± 1.775
5	55	24.1	32.9	28.46 ± 1.699
6	27	25.3	33.8	29.54 ± 1.898
7	12	28.4	34.3	30.28 ± 1.736
8	4	30.0	32.2	31.45 ± 0.998

* Average ± SD.

TABLE 4.

The average length at age and weight at age and the annual growth of *Chamelea gallina* calculated according to the Von Bertalanffy growth equation and the length weight exponential equation.

Age (y)	Mean Length (mm)	Annual Growth (mm)	Mean Weight (g)	Annual Growth (g)
1	15.3	15.3	1.14	1.14
2	20.8	5.5	2.77	1.63
3	24.7	3.5	4.56	1.69
4	27.3	2.6	6.09	1.63
5	29.2	1.9	7.40	1.31
6	30.5	1.3	8.40	1.00
7	31.4	0.9	9.14	0.74

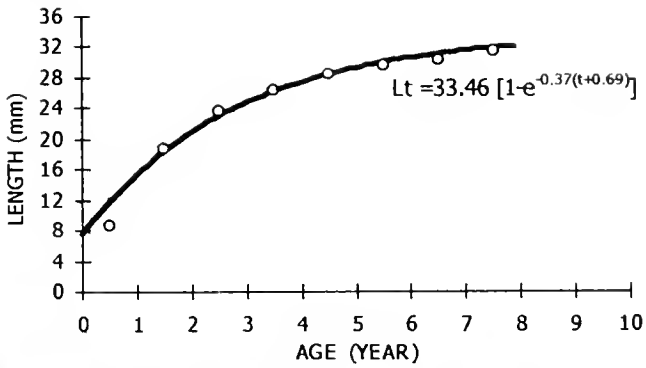

Figure 5. V. Bertalanffy growth curve of *Chamelea gallina* in the Northern Marmara Sea.

The investigations conducted on the biometric parameters in Turkey reported the maximum length of *C. gallina* as 36 mm in the Dardanellas (Alpaz & Önen 1989), 35.5 mm in northern Marmara Sea (Cebeci 1992), and 32.2 mm in western Black Sea coast (Deval 1991). Many studies performed on *C. gallina* showed a higher maximum length of the specimens sampled in Europe and in the Mediterranean. The maximum length of *C. gallina* was 41 mm in the coast of Spain (Vives & Suau 1962), 49 mm in the middle Adriatic Sea (Polenta 1993), and 46 mm in the southern Adriatic (Marano et al. 1982).

By analyzing the relationships between the weight and the dimensional parameters, calculated from regression and correlation analyses, the growth was determined to be allometric. The growth of *C. gallina* studied on the coast of Spain and in the sea of Adriatic was also allometric (Pogiani et al. 1973, Marano et al. 1982, Cano & Hernandez 1988).

The hyaline and the opaque growth bands were clearly evident in cross sections of valves from *C. gallina* from the Marmara Sea. These growth bands appeared to be formed seasonally and were used to age clams and to establish a length-at-age key. The parameters of the Von Bertalanffy growth equation calculated from these data, together with the size frequency distributions obtained from the monthly sampling program, show that *C. gallina* in the Marmara Sea has a lower growth rate and attains a smaller size in comparison with other Mediterranean areas.

In the Marmara Sea the annual growth pattern, characterized by reduced or no growth during the winter months with sea temperatures below 10°C, is similar to that reported for the Adriatic Sea (Froglia 1975, Polenta 1993, Arneri et al. 1995). In both areas a hyaline band is formed in the shell during winter months. In the western Mediterranean, off the southern coast of Spain, reduced growth and formation of the hyaline band were observed late in summer (Ramon & Richardson 1992), and apparently temperatures above 27°C negatively affect the growth of *C. gallina*.

Table 5 lists the average lengths at age reported in the literature for different Mediterranean locations. Although different authors used different methods to study the age and growth of *C. gallina*, the results obtained in different Adriatic regions are in agreement that, in the Mediterranean Sea, the most growth occurs in the first four age classes. The maximum sizes recorded by Adriatic workers are well above those reported for Spanish and Turkish *C. gallina* populations.

The differences in the growth rates of *C. gallina* off the Spanish, Adriatic, and Marmara coasts could be related to environmental factors such as annual sea temperature and salinity. *C. gallina*

TABLE 5.

Average lengths at age of *Chamelea gallina* reported in the literature for different Mediterranean localities.

Reference	Region	Method	Age (y)								Maximum Length
			1	2	3	4	5	6	7	8	
Poggiani et al. (1973)	Middle Adriatic	Surface rings	15	24	30	33	35				46
Froglia (1975)	Middle Adriatic	Length distribution	17	25							
Polenta (1993)	Middle Adriatic	Internal bands	17	25	30	34	38	39	42	45	49
Marano et al. (1982)	South Adriatic	Surface rings	15	23	31	35	38	42			46
Vives and Suau (1962)	West Meditter.	Surface rings	18	24	28	31					
Ramon and Richardson (1992)	West Meditter.	Internal bands	20	25	28	31					
Present investigation	North Marmara	Internal bands	15	21	25	27	29	30			34

has the highest growth rate in the Adriatic eutrophic waters. The lowest growth rate was observed in the population of the northern Marmara Sea (off the Tekirdag coast). Water masses of low salinity flow from the Black Sea to the Mediterranean Sea through the Marmara Sea (Kocatas et al. 1993). Therefore, the salinity over the Chamelea fishing grounds in the Marmara Sea (17–24‰) is significantly lower than in Adriatic and Spanish coastal waters (32–36‰). At the moment no experimental data are available to relate the different halide regimes to the observed differences in growth.

In conclusion, *C. gallina* reaches 15 mm at the end of the first year and 21 mm at the end of the second year. The regulations on the fishery of this species restrict the catch of individuals less than 22 mm (corresponding to 24 mm in length), and *C. gallina* reaches this height around the end of the third year (Fig. 5). The estimated growth coefficient and asymptotic maximum size were 0.37 and 33.46 mm, respectively.

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DISTRIBUTION, ABUNDANCE AND SOME POPULATION CHARACTERISTICS OF THE OCEAN QUAHOG, *ARCTICA ISLANDICA* (LINNAEUS, 1767), IN THE MECKLENBURG BIGHT (BALTIC SEA)

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ABSTRACT In the Mecklenburg Bight (western Baltic Sea), the ocean quahog, *Arctica islandica*, constitutes the most important species below the halocline. In 1999 a benthos survey at 95 stations in the Mecklenburg Bight showed a wide distribution of *A. islandica* at depths between 15.6 and 29.6 m. Mean abundance at these depths was 91 ind./m^2 with a biomass (AFDW) of 15 g/m^2. Maximum densities observed at these depths were 571 ind./m^2 and 120 g/m^2, respectively. The Mecklenburg Bight had an estimated colonized area of 5200 km^2 and a total annual production of 26500 t AFDW. In comparison to a data set of the 1960's, we found an increased quahog population. Ocean quahogs ranged from 1.5 to 64 mm in shell length and from 1 to 70 years of age. Growth was relatively slow for the first 40 years. Most (90%) individuals in the population measured <30 mm shell length, indicating strong recruitment in the Bight during the past decades. While the highest abundance (most juveniles) was observed in depths between 15 and 20 m, maximum biomass (due to adults) lay between 20 and 25 m. Probably the recruitment zone was displaced from below 20 m to 15–20 m depth in the last decades. Mean quahog wet meat yields was 18.3%. The individual ash free dry weight decreased significantly with increasing shell length from 14.3% (<10 mm) to 9.4% (>40 mm). All results were compared with data from populations in North Atlantic and adjacent waters.

KEY WORDS: *Arctica islandica*, ocean quahog, distribution, abundance, growth, size, meat yield, Baltic Sea, Mecklenburg Bight

INTRODUCTION

The ocean quahog, *Arctica islandica*, is an arctic-boreal species that occurs in North Atlantic and adjacent waters. In the North Sea region, the species' range extends into the Western Baltic Sea and reaches its eastern limit of distribution in the Arkona basin (von Oertzen and Schulz 1973). The largest populations reside in Kiel and Mecklenburg Bights. The ocean quahog is the largest bivalve in the Baltic Sea and lives for more than several decades (Arntz and Weber 1970, Brey et al. 1990). The long living species is an important indicator for environmental conditions. Beside salinity and sediment structure, oxygen concentration has a strong influence on the composition of Baltic Sea fauna and flora. Since the 1960's, oxygen deficiency below the halocline (16 to 20 m) has resulted in destruction of the bottom fauna of Mecklenburg Bight (Gosselck et al. 1987, Schulz 1968). Although *A. islandica* is highly resistant to oxygen depletion, the population is diminished by mortality caused by frequent and long lasting periods of anoxic conditions. In the deeper parts of Lübeck and Mecklenburg Bights a severe decrease of the Baltic Sea population of *Arctica islandica* has resulted during recent decades (Gosselck et al. 1987, Schulz 1968). Long living species like the ocean quahog (*Arctica islandica*) decreased in abundance and were replaced by communities of short living polychaetes (spionids, capitellids).

The geographical variability in growth rates of *A. islandica* is well documented for the North Sea and adjacent waters (Witbaard et al. 1999) and for US and Canadian east coasts (Kennish et al. 1994, Kraus et al. 1992, Murawski et al. 1982). Much is known about the reproduction, meat yield and age mostly for Atlantic populations off North America and Iceland coasts (e.g. Chaisson and Rowell 1985, Fritz 1991, Steingrimsson and Thorarinsdottir 1995, Thorarinsdottir and Einarsson 1996). Little is known about populations of this species in the vicinity of the Baltic Sea (Arntz and Weber 1970, Brey et al. 1990). The purpose of this study was to investigate the distribution, frequency and biomass of the recent population of *Arctica islandica* in Mecklenburg Bight as the first extensive study on the population characteristics of this important indicator species near its limit of distribution. A further aim was to compare these results with existing data from the 1960's compiled by Schulz (1969a) and to include an 11-year time series of one monitoring station.

Area of Investigation

The Mecklenburg Bight is part of the Belt Sea and belongs to the transition area between the North Sea and Baltic Sea (Fig. 1). It is connected with Kiel Bight via Fehmarnbelt and with Kattegat via the Belts. To the East, the Kadet trench crossing the Darsser rise connects it with the Baltic proper.

The location in the southwestern part of the Baltic with its relatively high salt content is decisive for benthic colonisation. Fauna and flora of the area are determined by hydrological and morphological factors, with salinity being the formative influence. Another important factor is the sediment structure, which is formed by residual sediments and mud.

During 1999 a macrozoobenthos survey was made in the Mecklenburg Bight. In total 95 stations were sampled between March and September (Fig. 1). The depth interval was 5 to 29.6 m, the sediment varied from fine sand at the shallowest stations to sand mixed with silt and clay at deepest stations. The sediment characteristics and current data within the area were published by Lange et al. (1991).

MATERIALS AND METHODS

Profiles of salinity were recorded throughout the water column using a CTD (conductivity/temperature/depth probe) system.

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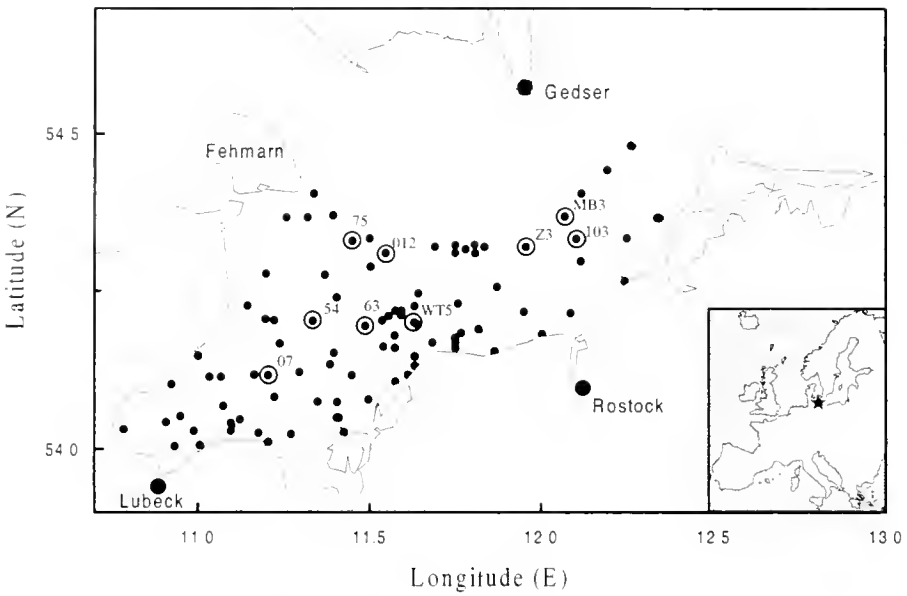

Figure 1. Investigation area with 95 stations in the Mecklenburg Bight (encircled stations refer to text and figures).

Samples for bottom water oxygen were taken with a 5 l water sampler (mounted on the CTD) at 0.5 m above the bottom and were determined with Winkler titration. Benthic samples were taken with a 0.1 m^2 Van Veen grab. At each station three parallels of grab samples were carried out. Due to sediment conditions grabs of different weights were used. The samples were sieved through a 1 mm screen and animals preserved with 4% formaldehyde in the field. For sorting in the laboratory, a stereo microscope with 10-40× magnification was used. A dredge haul (net mesh size 5 mm) was taken in order to obtain *A. islandica* specimens for the study of population size structure. For the characterisation of the habitats, i.e. assessment of sediment structure, epibenthos and current, an underwater video-system was used which was mounted on a sledge. The sledge was towed over the bottom by a drifting vessel at lowest possible speed (< 1 knot). The camera was installed on a pan and tilt head. Scaling was accomplished by four crossed laser beams projected into the picture. In addition bivalve siphon openings detectable at the sediment surface were counted to assess patchiness and distribution of adult (approximately >30 mm shell length) ocean quahogs.

At each station the shell length of all collected individuals of *A. islandica* were measured with a vernier calipers to the nearest 0.1 mm for the length-frequency distribution and the length-meat weight relationship. In total about 2300 specimens were measured. The valves and the wet meat of the specimens were weighed separately. Furthermore, the dry weight (DW) and ash free dry weight (AFDW) were determined to the nearest 0.01 mg. Because of very low weights, quahogs with shell lengths <3 mm were weighed in groups of 5 or 10 individuals sorted in size classes of 0.1 mm. Length-frequency distribution for each station was calculated for 10 mm size classes. The age of each *A. islandica* was estimated by measuring internal growth bands in the shell. This method is only applicable for specimens younger than 40 years (nearly all observed individuals are younger). For comparison and calibration, several shells were processed for observation of internal growth lines in acetate peels (see Ropes 1985).

The distribution map of the ocean quahog in the Mecklenburg Bight was made using Surfer (Win32) version 6.04 program of Golden Software Inc. The recent distribution was compared with the results from Schulz (1969a), whose data were transformed into the Surfer program to obtain a comparable map. Quahog distributional data from the monitoring station 12 of the Baltic Sea Research Institute Warnemuende and the Institute of Marine Research Kiel were used for the 11-year time series (1988–1999).

RESULTS

Bottom Water Variables

Salinity ranged between 7.5 and 22 psu in the water column. Bottom water salinity of areas inhabited by *Arctica islandica* varied between 12.5 and 22 psu in 1999. No oxygen deficiency was observed during our survey. Up to a depth of 16 m more than 7 mg/l were measured. Oxygen content decreased to a minimum of 4.5 mg/l only in the deepest parts of the Bight.

Distribution, Abundance and Biomass

In 1999 *Arctica islandica* was distributed between 15.6 and 29.6 m in the Mecklenburg Bight. Most stations within these depths were colonized (Fig. 2). Only in the deepest parts of the innermost area (Lübeck Bay) were no quahogs found. Furthermore, the outer Kadet trench with strong currents and stony substrates was not inhabited. The highest abundance was found in the south-eastern part of the Bight with a maximum of 571 ind./m^2 and an AFDW of 34 g/m^2 at station 103 (water depth 17.4 m). Thirty years ago (in the mid 1960's), *A. islandica* reached an abundance between 10 and 100 ind./m^2 with a maximum of 200 ind./m^2 in the central part of the Bight (Schulz 1969a) (Fig. 2). At depths below 20 m of the innermost area (Lübeck Bay), no *A. islandica* were found. The highest biomass (AFDW) was observed in deeper parts of the central Bight at station 54 (water depth 23 m) with 120 g/m^2 and an abundance of 236 ind./m^2 (Fig. 3). The Mecklenburg Bight had an estimated colonized area of 5200 km^2 within water depths of 15 and 30 m, a mean abundance of 91 ind./m^2 and a biomass (AFDW) of 15 g/m^2. Based on these values, the whole population

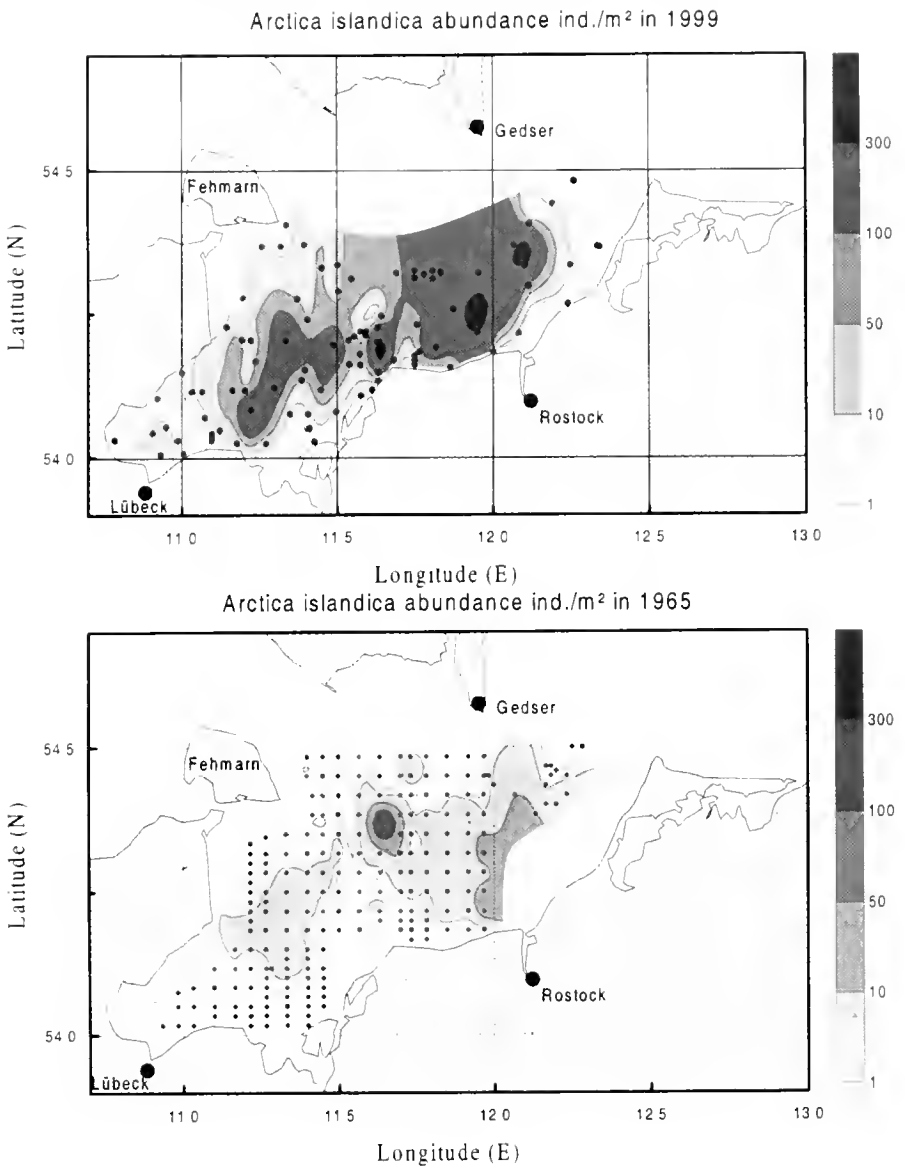

Figure 2. Distribution of *Arctica islandica* (ind./m²) in 1999 and during the investigation period in the mid 1960's of Schulz (1969a) (same scale for better comparison).

of the Bight was estimated at approximately 4.7E + 11 individuals with a biomass (AFDW) of 7.8E + 04 t (1.04E + 06 t wet weight).

In 1965 the mean abundance reached 20 ind./m². In the last 11 years the abundance of *A. islandica* at station 12 varied between 20 and 120 ind./m² (Fig 4). *A. islandica* occurred in mean density of 40 to 75 ind./m². However, due to patchy distribution of this species, the standard deviation was very high. The same was observed in 1999, when the highest abundance occurred with 120 ± 70 ind./m². Similarly, records by the video showed a similar patchy distribution of the quahog siphon openings. If only adults, specimens were taken into account, the average counts of openings were comparable with the estimated abundance by grabs at several stations. However, due to patchiness, the maximum abundance was much higher and ranged between 400 and 700 ind./m². Between these colonized centers, big patches of unsettled sediments were visible.

In this study the highest abundance was observed in depths between 15 and 20 m, whereas the maximum of biomass occurred between 20 and 25 m (Fig. 5). With increasing water depth, the abundance decreased from an average of 155 ind./m² in 15-20 m, to 85 ind./m² in 20 to 25 m, and 35 ind./m² in 25 to 30 m, respectively. In comparison the biomass (AFDW) was low in both the 15 to 20 m, and 25 to 30 m interval (7–9 g/m²), but averaged 28 g/m² at 20 to 25 m.

Population Structure, Meat Yield and Growth

During the benthos survey, shell lengths of *Arctica islandica* were between 1.5 and 64 mm (Fig. 6). Quahogs in the size class 0 to 10 mm composed 40% of the population. Particularly at shallower stations (15–20 m), the one- or two-year size classes (<10 mm) dominated. In deeper water (>20 m) individuals >20 mm were the dominant group. Generally only one size class dominated the population structure of an area. At shallow stations (stns WT5, MB3 and 103, see fig. 1 and 6) the most successful settlement took place during the last two years (the size class 0 to 10 mm domi-

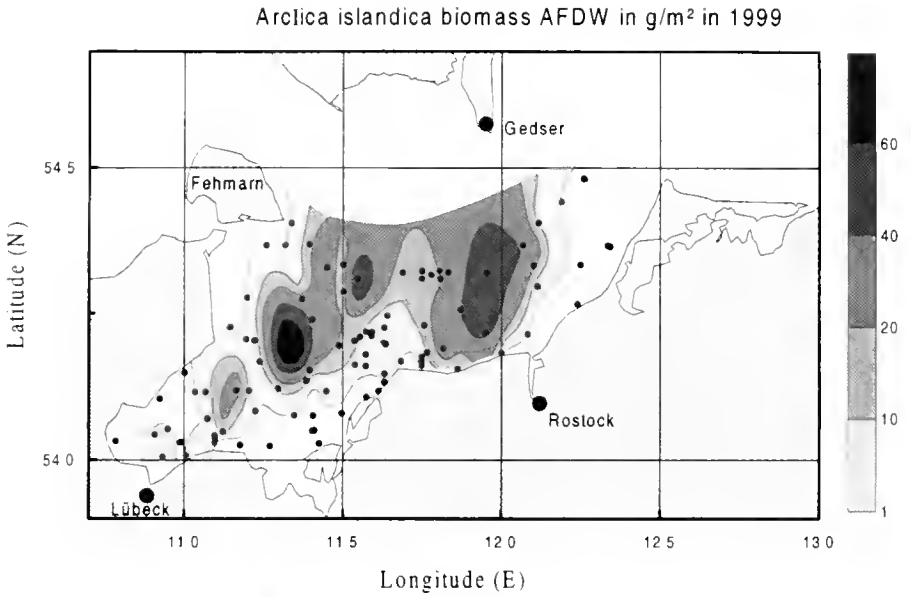

Figure 3. Biomass distribution of *Arctica islandica* (g AFDW/m²) in 1999.

nated with over 50%), whereas little or no recruitment was detected in stations deeper than 20 m. The dominant size class varied from 11 to 20 mm at stations 12 and Z3 via 21 to 30 mm at stations 63 and 07, to 31 to 40 mm at stations 75 and 54 (Fig. 6).

The wet meat yield (percentage of total wet weight) increased with increasing size up to shell length of 55 mm (Fig. 7). The mean meat yield of all individuals (1.5–55 mm) was 18.3% and varied between a minimum at 5% (2.7 mm shell length) and a maximum at 38% (47 mm shell length).

The organic content (all organic material of a quahog, i.e. meat, periostracum and ligamentum) was calculated as the relation of individual ash free dry weight to dry weight (Fig. 8). With increasing shell length a significant decrease in organic content was observed. The highest amount (14.3%) appeared in the shell size class <10 mm. The lowest organic content was 9.4% in the size class >40 mm. The mean organic content of DW of all individuals (1.5 to 55 mm shell lengths) ranged from 7.1% to 22.3% with a mean value of 11%.

Relationship between shell length and individual weight (meat wet weight and ash free dry weight) is shown in Figure 9. In these graphs all measured specimens (1.5–64 mm) of all stations are included. The smallest meat wet weight was 0.095 mg at a shell length of 1.5 mm and the largest was 14.7 g at 64 mm (Fig. 9a). The average meat yield per unit shell length of *Arctica islandica* is indicated by the estimated mean regression line. Differences between the station means were not statistically significant. The ash free dry weight varied between 0.1 mg (1.9 mm in length) and 1.6 g (64 mm in length) (Fig. 9b). The estimated regression lines of the different populations (stations) did not differ significantly. The results indicate that quahogs throughout the Mecklenburg Bight approximately contained the same meat per unit shell length for the range of length considered.

Growth was estimated by measuring internal growth bands in the shell. The corresponding equation for the calculated best-fit regression lines for all included individuals (1.5 to 55 mm) given in Figure 10. The oldest measured specimen reached an age of

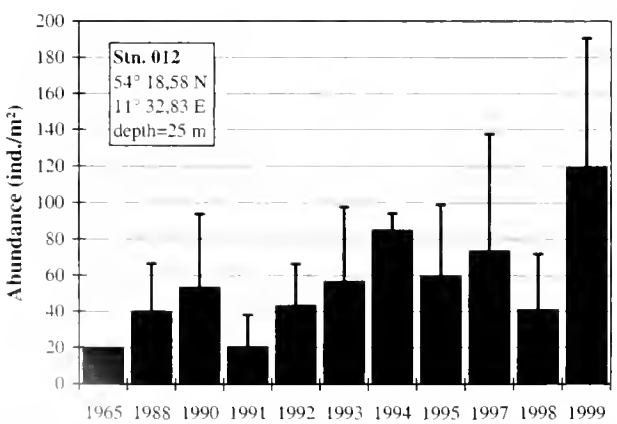

Figure 4. The development of mean abundance (± SD) of *Arctica islandica* at station 12 in the last 11 years in comparison with data of (Schulz 1969a) from the mid 1960's.

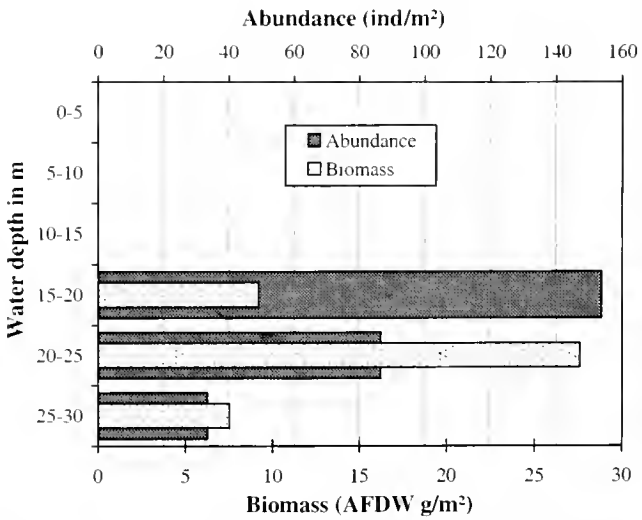

Figure 5. The vertical distribution of abundance (ind/m²) and biomass (AFDW g/m²) of *Arctica islandica* in 1999.

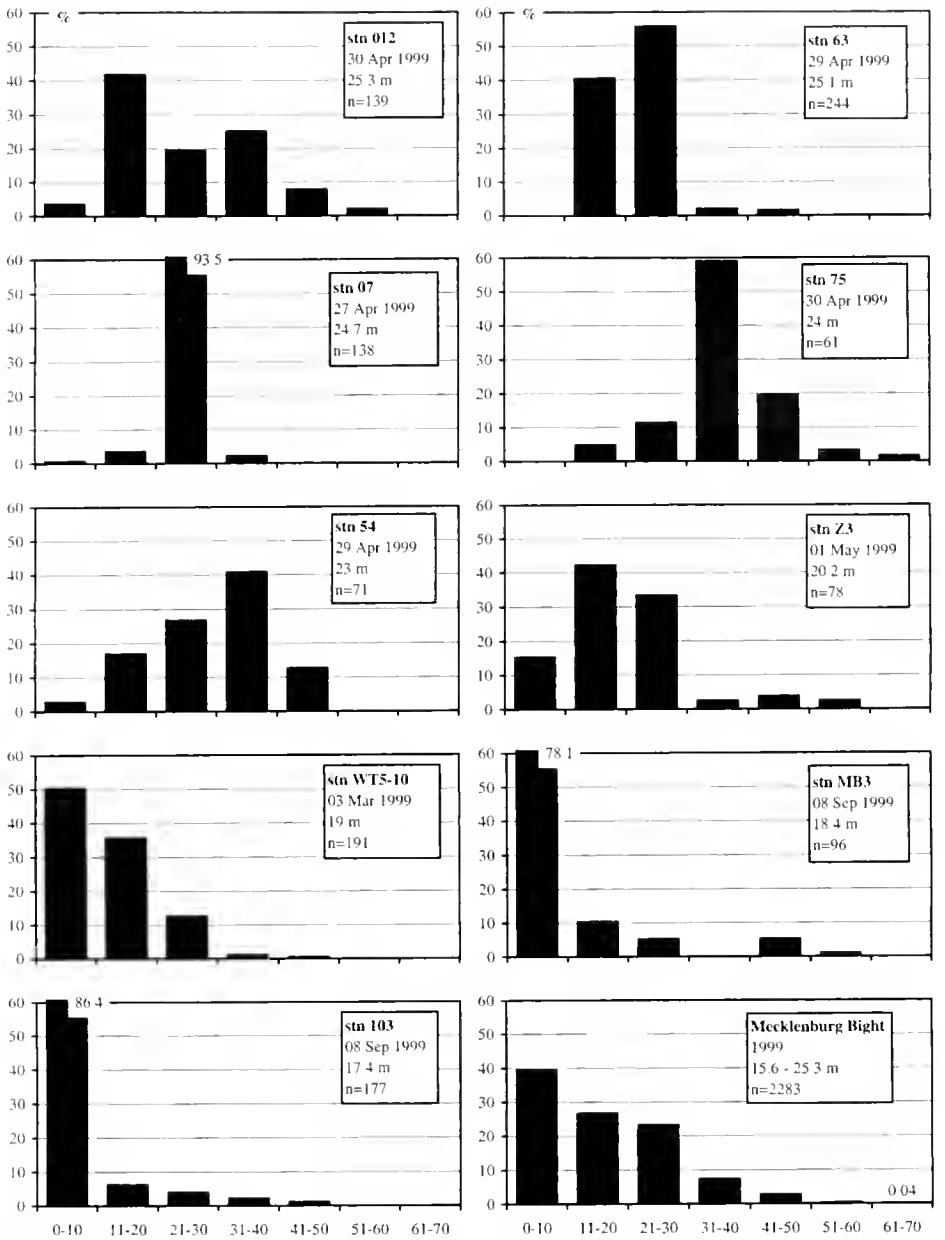

Figure 6. Shell length-frequency distribution for 10 mm size classes of *Arctica islandica* at several stations in depths between 17.4 m and 25.3 m in Mecklenburg Bight in 1999.

approximately 40 years. The largest and oldest specimen (64 mm) was not included because of difficulty of age estimation. After the equation, we got an age of app. 70 years.

DISCUSSION

The occurrence of *Arctica islandica* in the Mecklenburg Bight has been known since the last century when the first investigations on benthic fauna of the Baltic Sea took place (Boll 1852, Lenz 1875). While the main Baltic populations live in the Bay of Kiel and in parts of Sound and Belt, the distribution in the Mecklenburg Bight and the Arkona Basin represent the most eastern occurrence of this species in the Baltic Sea (von Oertzen and Schulz 1973). Due to the decreasing salinity in areas east of Mecklenburg Bight *A. islandica* has its natural limit of distribution there. Several investigations on biology and ecology of *A. islandica* of the Kiel Bay have been conducted (e.g. Arntz and Weber 1970, Brey et al. 1990, Oeschger and Storey 1993). However, information from the Mecklenburg Bight is very scarce (e.g. Al-Hissni 1989, Köhn 1989, Prena et al. 1997). Hagmeier (1930) observed a high biomass of *A. islandica* (195.1 g/m^2 fresh weight) in the Mecklenburg Bight during the 1920s, which is comparable with present biomass estimations. He found mostly adult specimens with juveniles scarcely occurring. The investigations of Schulz (1969a, b) at the end of the 1960s give the distribution pattern of *A. islandica* in the Mecklenburg Bight which was used for comparison with the present study. In the 1960s densities were low (20 ind./m^2 in mean). During the 1980s the abundance of the ocean quahog decreased in this region due to a long period of oxygen depletion (Gosselck et al. 1987,

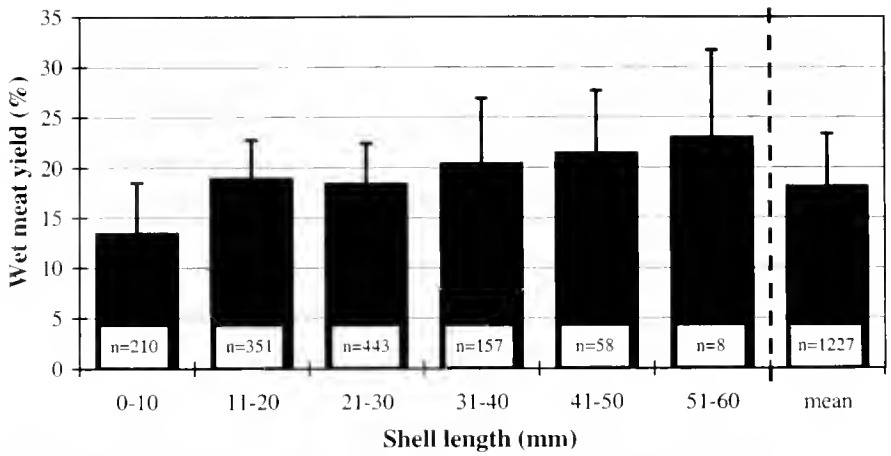

Figure 7. Mean wet meat yield in 10 mm size classes of *Arctica islandica* from the Mecklenburg Bight in 1999 (± SD).

Prena et al. 1997). In the inner part of the Lübeck Bay, in the 1980s no quahogs were found. In the early 1990s, the density of *A. islandica* increased in some regions of the Mecklenburg Bight (Al-Hissni 1989, Köhn 1989, Prena et al. 1997). Recently, a mean abundance of approximate 91 ind./m^2 in depths between 15 and 30 m occurred. The highest abundance was observed in the region between 15 and 20 m, whereas the maximum biomass occurred in deeper zones. Below the halocline, *A. islandica* is the most important species in the Mecklenburg Bight with respect to biomass. The mean biomass reached 15 g/m^2 AFDW (200 g/m^2 fresh weight). Brey et al. (1990) gave a P/B ration of 0.34 for populations in the Kiel Bay. If this ratio is applicable for the Mecklenburg Bight, the average annual production (AFDW) below the halocline amounts to 5.1 g/m^2. The total production of the Mecklenburg Bight is approximate 26500 t AFDW per year.

The different maximum zones for abundance and biomass are probably a result of recruitment failure in the deeper anoxic regions. In the deeper parts, oxygen depletion in late summer prevents the successful recruitment and growth of the juveniles, which settle mostly in summer and fall (Mann and Wolf 1983, von Oertzen 1972). The high biomass in the deep regions is based on adults (low numbers) that are able to survive critical times of oxygen depletion. *Arctica islandica* is one of the most tolerant species to oxygen deficiency and to hydrogen sulphide (von Oertzen and Schlungbaum 1972, Oeschger and Storey 1993, Theede et al. 1969). Nevertheless, Weigelt (1991) and Schulz (1969b) stated that possibly the high tolerance to oxygen deficiency is restricted to the adults, whereas the juveniles are more susceptible. Therefore, recruitment takes place only in favorable years. In the shallower zones (between 15 and 20 m), no major hypoxic episodes have been observed in the recent past (Matthäus et al. 1999). Although recruitment can take place in the shallower areas, conditions for growth are sub-optimal, probably due to the lower nutrition supply, lower salinity and higher temperature. The optimal temperature-range for fertilization, growth and survival of larvae and juveniles is 10° to 15° C (Landers 1976). Possibly the growing eutrophication of the western Baltic Sea explains the increased *A. islandica* population (see Arntz & Weber 1970, Persson 1987) and the displacement of the recruitment zone from below 20 m to the 15 to 20 m depth region.

The largest quahogs found were 64 mm long. In the 1960's Schulz (1969b) pointed out that only few specimens between 60 and 100 mm occurred in the central part of the Bight and juveniles were only found around 18 m depth. In this study sizes above 70 mm were observed only as empty shells. The most frequent size classes were 1.5 to 30 mm which included >90% of the *A. islandica* population of the Mecklenburg Bight. This population structure is completely different from populations of the Atlantic Ocean, where the smaller size classes are mostly absent (e.g. Fritz 1991, Murawski et al. 1982, Thorarinsdottir and Einarsson 1996, Steingrimsson and Thorarinsdottir 1995). We attribute this to successful recruitment in the Mecklenburg Bight during the last 10 to 15 years. In contrast in Atlantic waters, ocean quahog populations have only sporadic recruitment and strong recruitment events occur only every twenty (or more) years (Murawski et al. 1982, Steingrimsson and Thorarinsdottir 1995).

The mean wet meat yield of *A. islandica* of 18.3% (5% to 38%) is similar to other reported values. For a population in the Kiel Bay, Arntz and Weber (1970) reported a percentage of 19.3 to 22.2 with minimum values in winter. The meat yield of *A. islandica* off Iceland coasts ranged between 17% and 40% and averaged 30% (Thorarinsdottir and Einarssson 1996). If only the smaller size classes (<40 mm) of quahogs off Iceland are considered, then the mean wet yield of approximately 20% is similar to the result of this study. The meat yield observed from grounds off the coast of the United States and Canada ranged between 20.7% and 23% (Bakal et al. 1978, Chaisson and Rowell 1985).

In the Mecklenburg Bight, the mean individual ash free dry weight of *A. islandica* decreased from 14.3% to 9.4% with increasing size classes. The increasing mean wet yield and the decreasing

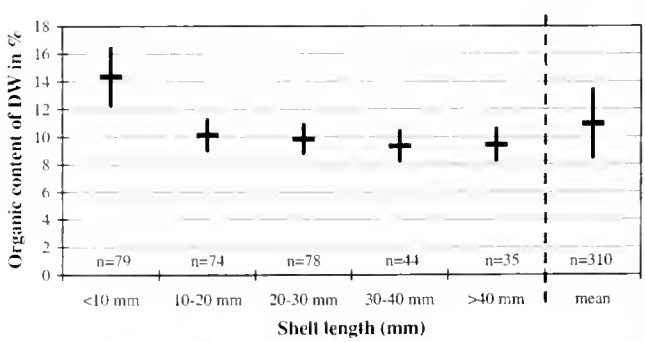

Figure 8. Mean individual percentage of ash free dry weight for different size classes of *Arctica islandica* from the Mecklenburg Bight in 1999 (± SD).

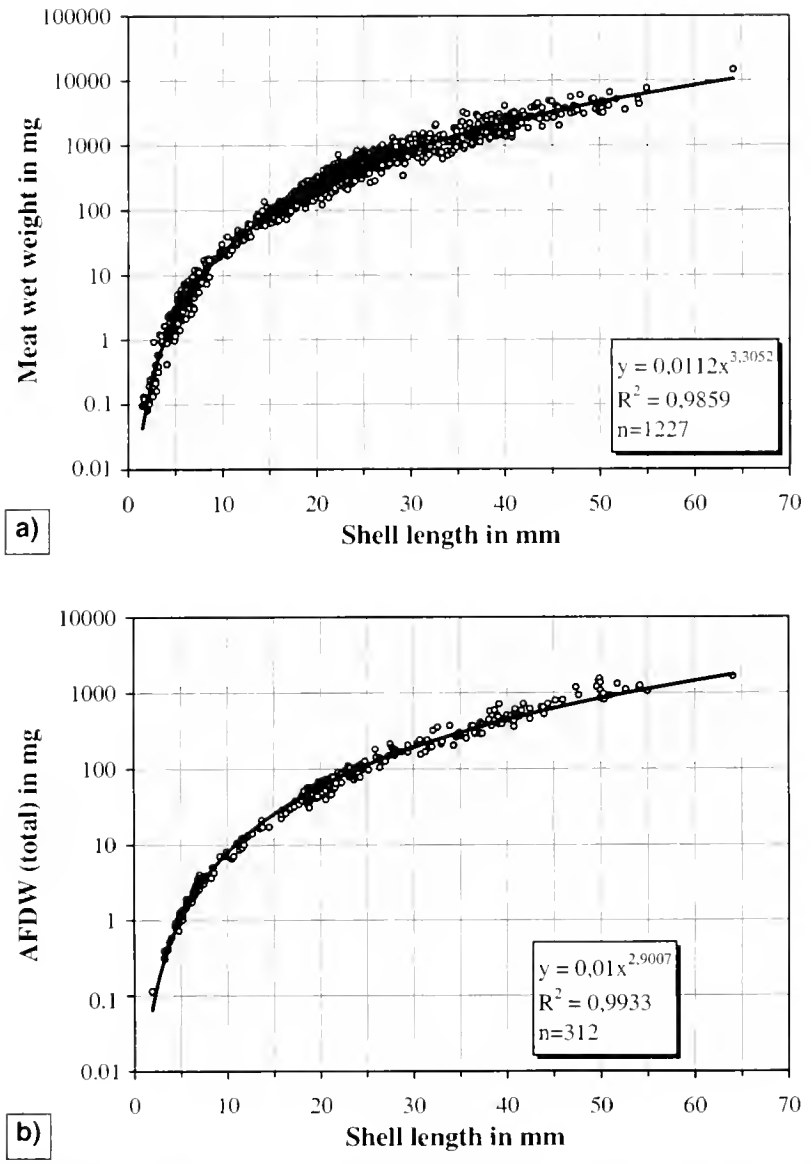

Figure 9. a) Estimated shell length-meat wet weight relationship for *Arctica islandica*, b) estimated shell length-ash free dry weight relationship for *Arctica islandica*. In these graphs all measured specimens of the whole Mecklenburg Bight populations are included. The corresponding equations for the calculated best fit regression lines is given in the figure.

ash free dry weight with increasing shell length indicate relatively higher water content of bigger quahogs. The length specific meat wet weight (shell-length to meat-weight relationship) in this study was similar to that reported for ocean quahogs off United States coasts. In the Mecklenburg Bight, the calculated meat wet weight for an individual of 95 mm shell length was 39 g. The meat wet weight of *A. islandica* off New York reported by Murawski et al. (1982) were 36 and 38 g and off New Jersey 37.9 and 51.3 g (Fritz 1991). The biggest meat wet weights of *A. islandica* ever reported (47.6, 55.5 and 70 g) were found off Iceland by Thorarinsdottir and Johannesson (1996). The calculated AFDW for an individual of 95 mm shell length reached 5.4 g.

Several authors reported on geographical differences in growth rates of *A. islandica* in its distribution area and between field and laboratory populations (e.g. Brey et al. 1990, Fritz 1991, Josefson et al. 1995, Kennish et al. 1994, Kraus et al. 1992, Steingrimsson and Thorarinsdottir 1995, Witbaard 1996, Witbaard et al. 1999). Ocean quahogs are among the slowest growing and long-living marine bivalves (Murawski et al. 1982, Thompson et al. 1980). Growth rates for this species vary with respect to location (temperature, food supply, salinity, and abundance). Kraus et al. (1992) observed the fastest growth in the laboratory where *A. islandica* reached 54 mm from 9.6 mm within three years. In the laboratory the specimens grow to 37 mm within two years. In the field they need 26 to 33 years to achieve the same shell length. The comparison of the growth curve of the Mecklenburg Bight population with growth curves calculated by Witbaard et al. (1999) for 12 populations from the North Sea and adjacent waters shows similar growth increments for the first 40 years of life. For the first five years, the Mecklenburg Bight population had a growth rate very similar to populations of the Kattegat with approximate linear increase in size with age (Josefson et al. 1995). In comparison with

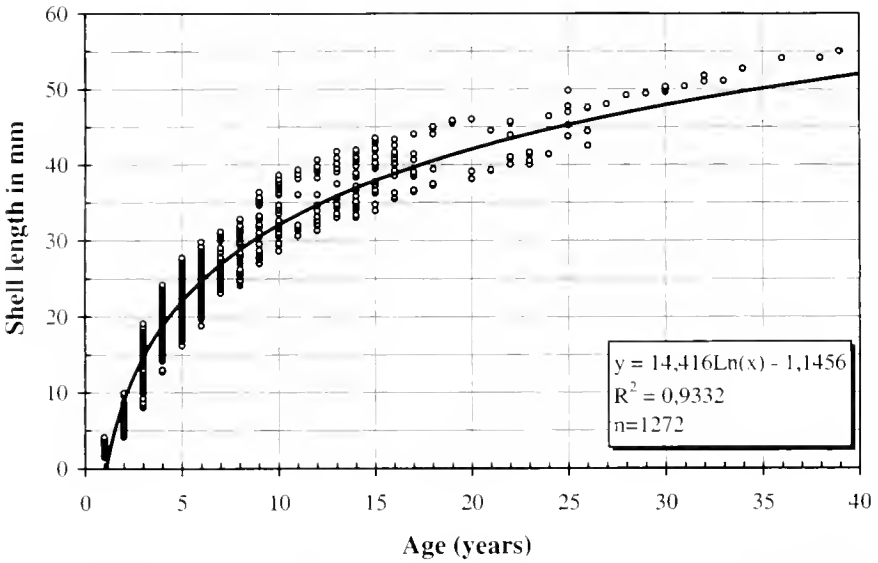

Figure 10. Growth curve of *Arctica islandica* from the Mecklenburg Bight for the first 40 years of life.

the growth rates of a Kiel Bay population calculated by Brey et al. (1990), the growth in the Mecklenburg Bight was slower. *A. islandica* in the Kiel Bay reached about 84 mm after 40 years; whereas, the specimens in the Mecklenburg Bight had a mean shell length of 52 mm in the same time. These differences are difficult to explain. The lower salinity may be one reason for the observed slower growth of quahogs in the Mecklenburg Bight. A possible further explanation might be that the Kiel Bay higher bottom currents (closer openings to the Small and Great Belt), enriched in suspended or re-suspended material, would supplement *A. islandica*, as a filter-feeder, with advective food supply (see Witbaard et al. 1999).

The present study shows the peculiarities of the ocean quahog in the Mecklenburg Bight near its eastern distributional boundary within the Baltic Sea. Further investigations must deal with the differences in observed growth rates of quahogs within the Baltic and adjacent waters. The comparative population dynamics of different water depths and/or sediment structure and the dispersion and settlement patterns of the larvae in this "border" area are of interest. Two of the main questions following this study are: Is the short life expectancy of quahogs in the Baltic due only to the salinity and which conditions cause the success of strong recruitment in its geographical distribution.

ACKNOWLEDGMENTS

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GROWTH, SURVIVORSHIP, AND NUTRIENT UPTAKE OF GIANT CLAMS (*TRIDACNA*) IN AQUACULTURE EFFLUENT

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ABSTRACT Experiments were conducted to evaluate the feasibility of using juvenile giant clams (*Tridacna*) to remove dissolved inorganic nutrients (nitrate and phosphate) from aquaculture effluent. Three (*T. derasa* Roding, *T. maxima* Roding, and *T. squamosa* Lamarck) of the four species tested during a two-month experiment all had very high survivorship in both effluent and control seawater, but *T. gigas* (Linne) experienced 50% mortality. *T. maxima* and *T. squamosa* showed virtually no growth in both effluent and control waters. *T. gigas* had the fastest growth rate among the four species. *T. derasa* grew significantly faster in effluent than in seawater. In a 24-h experiment (12 h dark followed by 12 h light), all the four species absorbed similar and significant amounts of nitrate and phosphate from effluent during the light period. Our study demonstrates that although it may not be possible to rely on giant clams alone to remove all excess dissolved nutrients from intensive food fish aquaculture effluent, it is entirely feasible to use giant clams to remove all excess nutrients in effluent of marine ornamental species. Giant clams can be either incorporated into a polyculture system with other marine aquarium trade species, or they can be grown in effluent in separate tanks.

KEY WORDS: aquaculture effluent, giant clams, growth, juvenile, nutrient uptake, survivorship, *Tridacna*

INTRODUCTION

In recent years, environmental impact and sustainable development have been central issues for aquaculture (e.g., Chua 1992, Pillay 1992, Shpigel et al. 1993, Shpigel & Neori, 1996). Many solutions have been attempted to remove the particulate and/or dissolved nutrients in aquaculture effluent. One way of alleviating aquaculture's environmental impact is to utilize recirculating system technology, thus reducing the amount of effluent introduced into the environment and allowing facilities to be located inland. Water exchange is most commonly used to prevent nutrient build-up in a recirculating system. The obvious drawback to this technique is the production of effluent, which must be disposed of under permitting restrictions. To completely eliminate effluent discharge, water from recirculating systems must be treated to remove particulate and dissolved waste before reintroduction to the culture system.

One of the techniques employed to treat effluent is to utilize micro- and macro-algal primary production to take up dissolved nutrients (e.g. Subandar et al. 1993). Suspended solids are usually removed by mechanical filtration. Biofiltration by bivalve mollusks has also been used to remove particulate waste from aquacultural effluent. In China, there is a long tradition of using polyculture and integrated culture to produce additional crops and reduce negative impacts on the environment (Yang et al. 1992). Integration of bivalve and/or seaweed culture to remove suspended particles and/or dissolved nutrients from aquaculture effluent has also been shown to be effective (e.g. Jones & Iwama, 1991, Shpigel et al. 1993).

Giant clams (family Tridacnidae) are unique among bivalve mollusks in that they possess symbiotic algae (*Symbiodinium microariaticum*) in their mantle tissue. In contrast to reef-building corals, the giant clams cultivate the symbiotic zooxanthellae in a special tubular system (Norton et al. 1992) which enables them to keep a substantially higher number of symbionts per unit area (Knop 1996). These algae contribute a variety of photosynthetically produced compounds to the host's metabolic needs (Lucas 1988). The single-cell thick tubes are bathed in haemolymph, allowing the efficient supply of nutrients to and photosynthate from the zooxanthellae (Belda & Yellowlees 1995). Klumpp et al. (1992) describe the tridacnids as "trophically opportunistic," capable of deriving nutrition using autotrophic or heterotrophic means.

Giant clams are endemic to the Indo-Pacific waters. The nine described species, *Hippopus hippopus* Linne, *H. porcellanus* Rosewater, *Tridacna crocea* Lamarck, *T. derasa*, *T. gigas*, *T. maxima*, *T. rosewateri* Sirenko and Scarlato, *T. squamosa*, and *T. tevoroa* Lucas, Ledua, and Braley, range from 10 cm to over 1 m in shell length (SL) as adults (Lucas 1988, Lucas et al. 1991, Knop 1996). They have formed a significant part of human diets in the Indo-Pacific and Southeast Asia for thousands of years. Clamshells have also been marketed. In recent years, giant clams have been successfully farmed for food, for restocking over-fished tropical reefs and, more recently, for the aquarium trade. The juvenile clams are popular in the aquarium trade due to their brightly colored mantle tissue, which is often exposed to allow light entry for algal photosynthesis. Their retail price ranged from $20 to over $200 per animal in the US. The brownish background coloration of the mantle is caused by the zooxanthellae, but the iridescent pigmentation creating different patterns in a variety of colors is not due to the algae (Knop 1996). This bright pigmentation may protect the clams against UV light or too much light in general and confuse potential predators (Knop 1996).

Tridacnids are ideal candidates for treating mariculture effluent. They have been successfully reared in captivity and are fast growing, reaching the 8–10 cm SL aquarium market size in only 2–3 years. Because of their unique trophic style, they could be successfully incorporated into recirculating mariculture systems to remove both nutrient and particulate waste. In addition, tridacnids are popular as aquarium pets and in the sushi/sashimi markets and would therefore be a valuable by-product.

We conducted a series of experiments to evaluate the potential of using the tridacnids for tertiary effluent treatment. The growth, survivorship, and nutrient uptake rate of four giant clam species growing in aquaculture effluent were compared.

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MATERIALS AND METHODS

Growth and Survivorship

In November 1996, juveniles of four giant clam species, *T. derasa*, *T. gigas*, *T. maxima* and *T. squamosa*, were obtained from a local aquarium dealer. The sizes were similar within a species, but considerably different among the species (Table 1), as constrained by the availability. The clams were imported from the Solomon Islands where they were farm-raised.

Upon arrival, the clams were acclimated to filtered seawater over a period of one hour. Clamshells were brushed briskly under running fresh water to remove any epiphytes and potential parasites. The clams were then introduced to two indoor raceways (245 cm long and 30 cm wide) filled with 15-cm deep seawater (110 L). Two metal halide lamps, 60 cm above the raceways, were used to provide sufficient light intensity and appropriate wavelength composition (Knop 1996). The light intensity maintained at 350–400 $\mu E/s/m^2$ and photoperiod was set at 12 hours light and 12 hours dark. The water temperature was maintained at 25–29°C, salinity at 35 ppt, and pH at 8.3, within the range for optimal growth for the giant clams (Lucas 1988, Price & Fagolimul 1988, Knop 1996).

After one week the clams were removed from the system, measured (SL) and tagged. The 48 clams were then randomly divided into the two raceways again (24 clams, six for each of the four species, in both the control and treatment raceways). For the following two months, 10% of the water in the raceways was replaced daily. In the control raceway, water was replaced with fresh filtered seawater; while the treatment raceway received effluent water from a fin fish aquaculture facility. Dissolved nutrient concentrations of the added effluent varied and were tested weekly, as were those of the filtered seawater, control and treatment raceways.

In the course of the two-month study period, biofouling became a problem, particularly in the treatment raceway. Two different species of parasitic snails were also found preying on the clams. *Cymatium muricium* feeds on the mantle of clams and the parasitic pyramidellid snails feed on clams' lymphatic fluid (Knop 1996, Boglio & Lucas 1997). To mitigate these problems, the clams were periodically removed from the raceways and brushed under running freshwater and returned to the raceways. Since most of the biofouling appeared to be algae, 25 herbivorous gastropods (*Astrea tecta*) were added to each raceway to control algal growth. Both techniques proved to be very effective. After two months of monitoring, all the clams were again removed from the raceways and measured (SL).

Dissolved Nutrient Uptake

A separate experiment was conducted to compare the dissolved nutrient uptake rates among the four species in January 1997. All the clams used in the experiment had been acclimated to effluent water for at least two weeks prior to the study. Only healthy looking clams with quick shell closing responses were used.

Each clam was immersed in a 500-ml beaker with 1-μm filtered effluent water of known nutrient concentrations of dissolved nitrate (NO_3) and phosphate (PO_4). Five replicate clams were used for each species. Again, the sizes were similar within a species, but considerably different among the species. Mean (±s.d.) SL for *T. derasa*, *T. gigas*, *T. maxima*, and *T. squamosa* are 60.2 (±2.2), 84.8 (±5.4), 32.4 (±1.7) and 36.6 (±2.3) mm, respectively. Two controls (each with a pair of empty giant clamshells) were set up. Nitrite and ammonia levels in the effluent were below detection limit (0.01 ppm) at the beginning and end of the experiment. The clams remained in darkness for 12 hours after which a sample of the effluent was removed from each beaker and tested for nitrate and phosphate concentrations. The clams remained immersed for an additional 12 hours under light with intensity of 350–400 $\mu E/s/m^2$. Samples of the effluent were again removed and tested during the 12-hour light period (every two hours for nitrate, at the end of the 12-hour light period for phosphate). The water temperature was maintained at 2–29°C, salinity at 35 ppt, and pH at 8.3.

Measurement of dissolved nutrient (ammonia, nitrite, nitrate, and phosphate) concentrations followed the techniques described in Menzel and Corwin (1965), D'Elia et al. (1975), and Greenberg et al. (1985). Ammonia was determined using the direct nesslerization method, nitrate the cadmium reduction method, nitrite the azo-dye formation technique, phosphate the persulfate oxidation method and the resulting orthophosphate be measured using the ascorbic acid method. Color development in all cases was measured using a spectrophotometer.

RESULTS

Growth and Survivorship

All the individuals of *T. maxima* and *T. squamosa* survived. One *T. derasa* in the treatment tank and three (50%) *T. gigas* in both treatment and control tanks died. *T. derasa* and *T. gigas* displayed some growth during the study, whereas *T. maxima* and *T. squamosa* showed virtually no growth (Table 1). Only *T. derasa* showed significant difference in growth ($P = 0.008$, *t*-test) between the treatment and control tanks (Table 1).

Concentrations of dissolved nutrients in the seawater and control tanks remained low (Fig. 1). Except for the first two weeks, concentrations of ammonia and nitrite in the effluent and treatment tanks were also low (Fig. 1), probably due to the nitrification process carried out by the biofilter in the fin fish culture facility. The concentrations of nitrate (Fig. 1c) and phosphate (Fig. 1d) in the treatment tanks were lower than those in the effluent tanks, suggesting that the clams were removing the nutrients throughout the two-month period.

TABLE 1.

Mean (±s.d.) initial SL and SL increase of the four clam species after the two months of immersion in control (C) and treatment (T) raceways.

	T. derasa		*T. gigas*		*T. maxima*		*T. squamosa*	
	C (n = 6)	T (n = 5)	C (n = 3)	T (n = 3)	C (n = 6)	T (n = 5)	C (n = 6)	T (n = 6)
Initial SL (mm)	53.8 ± 11.0	59.2 ± 1.6	86.0 ± 6.1	86.7 ± 7.5	33.2 ± 7.5	32.3 ± 1.9	35.6 ± 2.9	36.0 ± 3.0
SL Increase (mm)	0.2 ± 0.4	1.8 ± 1.1	1.3 ± 2.3	2.7 ± 2.1	0.2 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.4

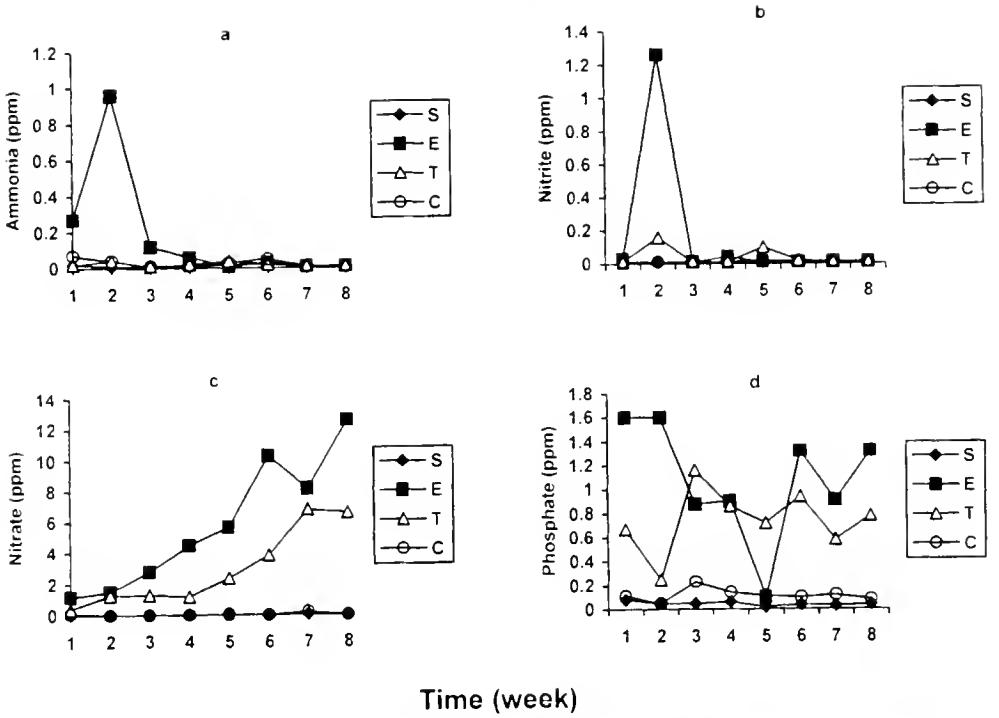

Figure 1. Concentrations of ammonia (a), nitrite (b), nitrate (c), and phosphate (d) in filtered seawater (S), effluent (E), treatment water (T), and control water (C) over the two-month study period.

Dissolved Nutrient Uptake

The concentrations of nitrate and phosphate did not change in the two controls over the 24-h period and in the treatments over the 12-h dark period (Fig. 2). All four species showed rapid uptake of both nitrate and phosphate during the 12-h light period (Fig. 2). Over the 24-h period, the nitrate concentration decreased from 12.6 ppm to an average (±s.d.) of 9.0 (±0.3) ppm for *T. derasa*, 8.0 (±0.2) ppm for *T. gigas*, 10.0 (±0.2) ppm for *T. maxima*, and 9.9 (±0.7) for *T. squamosa* (Fig. 2). Taking clam size into consideration, all four species had similar rates of nitrate uptake. The phosphate concentration decreased from 0.23 ppm to an average (±s.d.) of 0.15 (±0.01) ppm for *T. derasa*, 0.14 (±0.01) ppm for *T. gigas*, 0.14 (±0.00) ppm for *T. maxima*, and 0.18 (±0.00) for *T. squamosa* (Fig. 2). Phosphate uptake rates were also proportional to clam size, except that *T. maxima* seems to be more efficient.

DISCUSSION

Klumpp et al. (1992) describe the tridacnids as "trophically opportunistic," capable of deriving nutrition using autotrophic and/or heterotrophic means. The ability of the giant clams to assimilate inorganic nutrients via photosynthesis has been well-documented (Wilkerson & Trench 1986, Fitt 1988, Mingoa 1988, Solis et al. 1988, Braley et al. 1992, Hastie et al. 1992, Klumpp et al. 1992, Belda et al. 1993b, Fitt et al. 1993, Klumpp & Griffiths 1994, Klumpp & Lucas 1994, Hawkins & Klumpp 1995). Autotrophy under high light intensities may be able to satisfy the basic nutritional needs of the clams (Griffiths & Streamer 1988, Klumpp et al. 1992, Klumpp & Griffiths 1994, Klumpp & Lucas 1994).

Increased growth in the giant clams with increasing dissolved nitrogen concentrations was reported in several studies (Wilkerson & Trench 1986, Solis et al. 1988, Braley et al. 1992, Hastie et al.

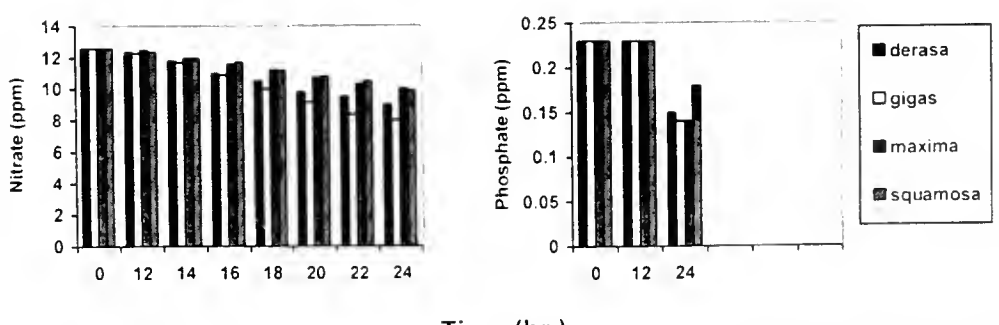

Figure 2. Mean nitrate and phosphate concentrations (ppm) over the 24-h period (12 h dark followed by 12 h light) for *T. derasa* (mean ± s.d. SL = 60.2 ± 2.2 mm), *T. gigas* (mean ± s.d. SL = 84.5 ± 5.4 mm), *T. maxima* (mean ± s.d. SL = 32.4 ± 1.7 mm), and *T. squamosa* (mean ± s.d. SL = 36.6 ± 2.3 mm).

1992, Fitt et al. 1993). In a 60-day nutrient enhancement study with *T. derasa* (Hastie et al. 1992), the clams grown in elevated dissolved inorganic nitrogen had both higher SL and tissue weight increments over untreated controls. Ammonium and nitrate were equally effective and no detrimental physiological effects of enrichment were detected. The clams (initial average size: 27.6 mm SL) in the elevated nitrate treatment grew an average of 0.1–0.16 mm SL/day, as compared to 0.06 mm SL/day for the control seawater (Hastie et al. 1992). In our two-month study, the much larger sized (average initial SL were 53.8 and 59.2 mm for control and effluent tanks, respectively) *T. derasa* grew an average of 0.03 mm SL/day in the effluent, but had negligible growth (0.004 mm SL/day) in the control seawater. A large-scale grow-out study at 11 village farms in Solomon Islands (Hart et al. 1998) found that among the juveniles (20–30 mm SL) of giant clam species tested, *T. derasa* had the best growth (0.14 to 0.21 mm SL/day) and survival (80.1 to 99.1%), and therefore the highest estimated revenue for the aquarium trade, than those of the other two species tested: *T. maxima* (growth: 0.07–0.14 mm SL/day, survivorship: 16.0–72.7%) and *T. crocea* (growth: 0.04–0.07 mm SL/day, survivorship: 11.8–81.9%). In a similar 8-month grow-out study on *T. squamosa* in the Solomon Islands (Foyle et al. 1997), the clams (initial size about 24 mm SL) grew an average of 0.08–0.29 mm SL/day with the survivorship ranged from 7–83%. Growth rates of juvenile *T. squamosa* found in other studies ranged from 0.05–0.23 mm SL/day (Klumpp & Griffiths 1994, Foyle et al. 1997). *T. gigas* has the highest growth rate (0.13–0.37 mm SL/day) among the giant clam species tested in our experiment and other studies (see Table 7 of Klumpp & Griffiths 1994 for a review summary). The average daily growth rates of *T. gigas* in the control seawater (0.02 mm SL) and in effluent (0.05 mm SL/day) in our study are much lower than those of the other studies. Interestingly, while effluent greatly enhanced the growth of *T. derasa* and probably *T. gigas*, it did not have an effect on the other two species: *T. maxima* and *T. squamosa*. In fact, these two species showed virtually no growth during the two-month period, in effluent as well as in control seawater (Table 1).

Giant clams are hardy animals and have excellent survivorship when grown in effluent in a recirculating system. Among the four species tested in both seawater and effluent in our two-month study, *T. gigas* was the only species that suffered significant (50%) mortality. However, available literature does not indicate that *T. gigas* is less hardy than the other species. Ranellids (e.g., several species of *Cymatium*) and pyramidellid snails are common predators/parasites of tridacnid clams in the wild and can cause serious mortality (Knop 1996, Boglio & Lucas 1997). The pyramidellid snails are obligate parasites with various degrees of host specificity. They use their proboscis to penetrate the hosts and gain their nutrition by feeding purely on the hosts' body fluid (Boglio and Lucas, 1997). The snails are small (a few mm) nocturnal feeders and can be difficult to detect and remove. Therefore, it is critical to carefully examine imported clams for parasites.

The four species tested in our study showed similar capabilities of absorbing both nitrate and phosphate, after taking clam size into consideration. The nitrate uptake rates found in our study are similar to those found in the studies of juvenile *T. derasa* grown on seawater spiked with artificial nutrients (Hastie et al. 1992, Fitt et al. 1993). Fitt et al. (1993) also found that the rate of NO_3 uptake was lower than that of NH_3. In fact, uptake of NO_3 was repressed in the presence of NH_3. In the present study, low NH_3 concentration in the effluent may have facilitated the uptake rate of nitrate by the clams. In the study of Fitt et al. (1993), no detectable uptake of ammonia and much lower uptake rate of nitrate in the dark as compared to under light were found. Among the four clam species we tested, virtually no uptake of nitrate or phosphate took place during the 12-h dark period (Fig. 2).

There has been limited study of phosphate uptake by giant clams. Large juvenile *T. gigas* (22–26 cm in SL) can take up significant amount of phosphate, with the individuals previously held in control seawater deplete phosphate at a much greater rate (11.5 μM P/hr.) than the ones previously held in phosphate-supplemented (10 μM) raceways (4.3 μM P/h) (Belda & Yellowlees 1995). The four species we tested, especially *T. maxima*, can rapidly absorb phosphate from seawater in the light period (Fig. 2). They are also capable of continuously removing phosphate for an extended period of time (Fig. 1d). Zooxanthellae do not have ready access to inorganic phosphate in seawater and within the clam (Belda et al. 1993b, Belda & Yellowlees 1995). The clam host may actively regulate P availability to its symbiotic algae (Belda et al. 1993b). Juveniles of *T. derasa* exposed to elevated phosphate (2 μM) alone actually grew less than control clams and phosphate additions to elevated dissolved inorganic nitrogen treatments had little apparent effect on the clam's growth rates (Fitt et al. 1993). High concentrations of phosphate inhibit calcification (Simkiss 1964) and may have a negative effect on the growth of giant clams. Further studies are needed to examine the effects of dissolved phosphate concentration on clam's growth if aquaculture effluent is to be used for culturing giant clams.

Elevated nutrients of inorganic nitrogen and phosphorus, however, may lead to weakening shell structure in the giant clams, resulting in thinner and more transparent shells (Belda et al. 1993a). Ammonium, as well as phosphate, may inhibit or depress calcification in clams (Simkiss 1964, Belda et al. 1993b). These clams may be more vulnerable to predation if released to the wild, but would not have any negative impacts when used in the aquarium trade. Enhanced nutrients also increase biofouling in the clams, but this can be removed by gentle scrubbing with a toothbrush (Hastie et al. 1992; present study) or by introducing appropriate grazers, such as herbivorous snails (present study).

The relative importance of autotrophy in giant clam nutrition has been the subject of speculation for many years (Trench et al. 1981, Fisher et al. 1985). The relative contributions of phototrophy and heterotrophy toward the carbon requirements vary among the tridacnid species and among different sized clams within a species (Klumpp et al. 1992, Klumpp & Griffiths 1994, Klumpp & Lucas 1994). *T. gigas* was the most efficient in uptake of C via both photosynthesis and filter feeding among the four species compared (*T. gigas*, *T. crocea*, *T. squamosa*, and *Hippopus hippopus*). The interspecific differences, however, declined with clam size (Klumpp & Griffiths 1994). Filter feeding is able to provide over half of the total carbon needed both for respiration and growth in small individuals of *T. gigas* (Klumpp et al. 1992). However, filter feeding is a relatively minor source of energy in other species (*T. crocea*, *T. derasa*, *T. squamosa*, *T. tevoroa*, and *Hippopus hippopus*) and its importance decreased with increasing clam size (Klumpp & Griffiths 1994, Klumpp & Lucas 1994). In the present study, the degree of nutritional contribution to the clams by small particles in the effluent is unknown.

Among the four species we tested, *T. derasa* is probably the best candidate for culturing in aquaculture effluent. In addition to growth, survivorship, and nutrient uptake parameters, seed availability and ease of cleaning to remove epiphytes and parasites also

need to be taken into consideration. All the four species are equally susceptible to epiphyte infestation, but those with smooth shells, such as *T. gigas* and *T. derasa*, are easier to clean by scrubbing. The highly fluted shells of *T. squamosa* and *T. maxima* are more likely to harbor parasites and other epifauna, which are harder to detect and remove due to the shell's high relief. Seed of *T. gigas* is becoming difficult to obtain as most hatcheries are concentrating on the more colorful species. All the four species are popular in the aquarium trade, with the more colorful *T. maxima* commanding higher prices.

It may not be possible to rely on giant clams alone to remove all the dissolved nutrients from intensive food fish aquaculture effluent at a commercial scale. However, it is entirely feasible to use giant clams to treat aquaculture effluent of other marine ornamental species. Most of more than 3,000 species of marine fishes and invertebrates marketed in the aquarium trade industry in the world are collected from coral reef systems. Concerns over the impacts of wild harvest, with expanding popularity of coral reef animals in the aquarium trade, have spurred interest in developing or improving cultivation technology for marine ornamental fish (Fletcher et al. 1999, Holt 1999), shrimp (see Lin et al. 1999 for a review), corals (Carlson 1999), giant clams, and other invertebrates.

Either giant clams can be incorporated into the other aquarium species in a polyculture system, if the environmental requirements are compatible between (among) the species; or they can be grown in effluent in separate tanks. The clams can be grown not only in tropical and subtropical regions, but also in greenhouses in temperate areas, as currently practiced by the aquarium industry. In colder areas and/or months, the effluent may be diverted to an indoor greenhouse to be treated by the giant clams (Braley et al. 1992). Growing juvenile giant clams in recirculated water would also pave the way for inland culture (Braley et al. 1992), thereby eliminating the possible negative consequence of introducing exotic species.

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REPRODUCTIVE CYCLE AND BIOCHEMICAL COMPOSITION OF THE ARK SHELL *SCAPHARCA BROUGHTONII* (SCHRENCK) IN A SOUTHERN COASTAL BAY OF KOREA

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ABSTRACT The reproductive cycle and biochemical composition of the ark shell *Scapharca broughtonii* (Schrenck) in Chinju Bay, a southern coastal bay of Korea, were studied from July 1999 to August 2000 in relation to environmental conditions (temperature, salinity and available food). Animals were cultured in the bottom. *S. broughtonii* from Chinju Bay was characterized by a spawning period during July to September due to a monocyclic gametogenesis throughout the year. Clear seasonal cycles in biochemical composition of the animal tissues were recorded. Summer spawning of *S. broughtonii* was followed by a clear accumulation period of reserve materials in autumn (from October to December). A great increase in animal tissue weight was attributed to the rapid accumulation of protein and carbohydrate during that period. A new cycle of gametogenesis began at the lowest water temperature in February. The gonadal growth during winter to spring when the available food was poor took place at the expense of reserves (mainly glycogen) accumulated previously in autumn. Lipid biosynthesis during gametogenesis was associated with glycogen breakdown. Blooms of phytoplankton in the ambient waters took place in the low saline summer period when water temperature was highest, depending on the addition of excessive nutrients via streams. Spawning of *S. broughtonii* occurred at the time when the available food was very abundant. This seemed to enable larvae to be produced at the most opportune moment with regard to food availability, as previously proposed. Our results indicate that this bivalve may be considered a typical conservative species in gametogenic pattern.

KEY WORDS: *Scapharca broughtonii*, reproductive cycle, bivalves, biochemical composition, seston, food index

INTRODUCTION

The southern coastal bay systems in Korea have been widely exploited for shellfish cultures. In the shallow semi-enclosed coastal bays, uncontrolled increases in stock density often result in reduced growth rates and environmental disruption thereby causing mass mortality of cultured bivalves (Park et al. 1998, Park et al. 1999). The ark shell *Scapharca broughtonii* (Schrenck) is cultured in the bottom of the inshore bay systems. Thus, this shell, which is a common benthic suspension feeder from intertidal to subtidal zone along the northwestern Pacific coast, is one of the most important commercial bivalve species in Korea. Morphological and genetic differences between the ark shell populations of Korea, China and Japan have been found (Lee et al. 1997, Yokogawa 1997). Annual production of the ark shell *S. broughtonii* in Korea was maximized in 58,000 tonnes in 1986. However, since the 1990s production has decreased, depending largely on local shortages of healthy seed (Rho & Pyen 1977, Park et al. 1998). Knowledge of reproductive strategy in a given area could have essential importance for collecting ark shell seed from nature.

Up to now, biological studies on the ark shell have been limited to natural and artificial seed collection and growth of spat (Kim & Koo 1973, Yoo & Yoo 1974, Pyon et al. 1976, Rho & Pyen 1977, Kim et al. 1979, Kim & Yoon 1980). Little attention has been paid to the physiological ecology and gametogenic cycle (Park et al. 1998). The reproductive cycles of marine bivalves are strongly related to energy storage-utilization cycles and environmental parameters such as water temperature and food availability (Giese 1969, Gabbott 1975, 1983, Bayne 1976). Therefore, seasonal cycle and frequency of the reproductive activity in marine bivalves exhibit geographical variations within a single species (Bayne & Worrall 1980, Newell et al. 1982, Rodhouse et al. 1984, Bricelj et al. 1987). Gametogenesis is an energy-demanding process. The use of energy required for gametogenesis differs between species. According to the classification of Bayne (1976), one group, called opportunistic species, uses the recently ingested energy from seston and another group, conservative species, uses the energy stored in various organs through feeding prior to its gametogenesis. The seasonal cycles of gametogenesis and reserve storage in *S. broughtonii* may reveal its reproductive strategy in relation to local environmental conditions.

This study presents the reproductive cycle and biochemical composition of *S. broughtonii* cultured in the bottom of Chinju Bay, a southern coastal bay system of Korea. The objective of the study was to observe the seasonal cycles of energy storage-consumption in the ark shell, to examine the use of reserve materials in relation to the gametogenic cycle and the environmental parameters, and to establish the gametogenic pattern.

MATERIALS AND METHODS

Study Site

Chinju Bay is a small shallow bay system (average depth 4 m) situated on the southern coast of Korea (Fig. 1). It is well protected by Namhae and Changsun Islands to the south. The bay is about 25 km long and 13 km wide. It is a well-mixed bay and the tide is semidiurnal with a maximum tidal range of 3.6 m and strong tidal currents. Freshwater pulses occur irregularly from an artificial dam of the Nam River on the north of the bay. Sediments of the bay are composed mostly of mud. A broad area (about 8 km^2) of its subtidal zone has been developed for bottom culture of the ark shell *S. broughtonii*. The sampling station for the study was located in the southwestern part of the bay. It was 3 m deep at the low tide and 6 m at the high tide.

Sample Collection and Preparation for Analysis

Fifty to 100 specimens of *S. broughtonii* were collected randomly by diving at monthly intervals from June 1999 to August

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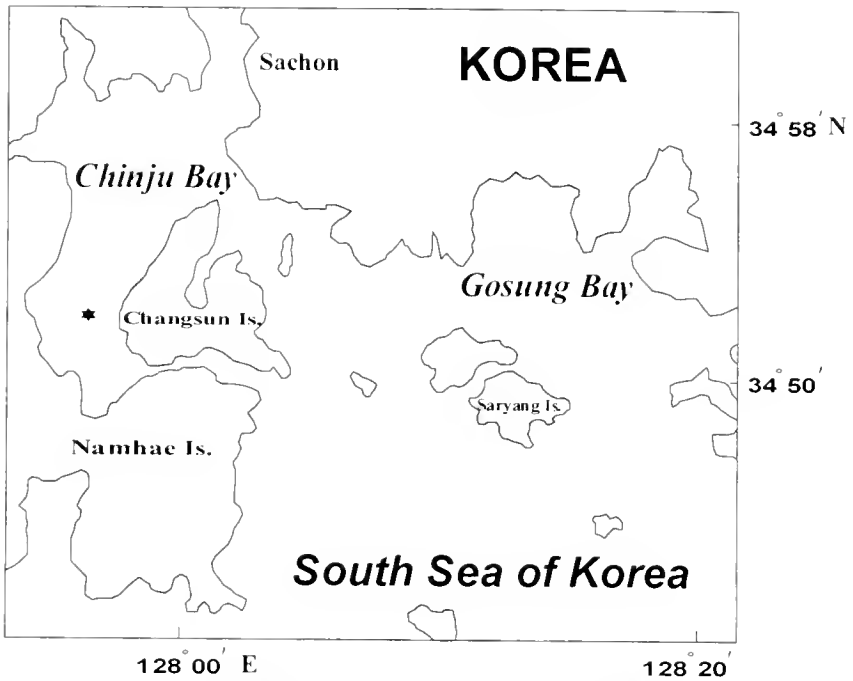

Figure 1. Location of the site for ark shell collection and environmental measurements in Chinju Bay, Korea.

2000. Mean shell length was 66.5 (± 6.2) mm for one-year class. These animals were seeded on the bottom in April 1998. All animals were carried to the laboratory quickly and kept alive overnight to evacuate gut content. After biometric measurements (shell length, width, height, and total weight) they were dissected carefully. The flesh was weighed for wet tissue weight and freeze-dried for the determination of dry tissue weight and biochemical composition. Shell valves were rinsed with distilled water and weighed after drying in a furnace at 50°C for 48 h. Temperature and salinity of the surface and bottom waters were recorded at the time of sampling by a CTD meter (Seabird Electronics, Inc.). We first investigated the characteristics and the seasonal variability of seston of the bay. Waters for seston measurements were pumped for the surface and bottom waters at the same time and screened through a 180-μm Nitex mesh to eliminate zooplankton and large debris. Water samples for suspended particulate matter (SPM) were immediately filtered through a preweighed Whatmann GF/C glass-fiber filter. For pigment and chemical analysis, 0.5 to 3-L aliquots were concentrated on GF/F filters on board *in situ* and the filters were carried to the laboratory. All filters were then kept frozen (−80°C) until analyzed.

Seston Analysis and Food Index

Filters for dry weight were dried at 80°C for 24 h and reweighed after cooling in desiccator. Chlorophyll *a* concentration was determined on acetone extracts using fluorometric method as modified by Parsons et al. (1984) with 10 AU Fluorometer (Turner Designs). Biochemical components of seston (particulate protein, carbohydrate and lipid) were analyzed by the same methods as described later for animal tissues. An evaluation of the nutritional value of the seston throughout the year was done using the biochemical composition. Food quantity was defined as the sum of the concentrations of these components and a food index was calculated as the percentage of food in the seston [(Food/Total Seston) ×100] (Widdows et al. 1979).

Condition Index and Reproductive Activity

Temporal variation of condition index reveals either the onset of accumulation of organic matter for reproduction or release of gametes. The condition of the ark shells was estimated from an index that was expressed as dry tissue weight:dry shell weight (Walne 1976, Mann & Glomb 1978). Thirty individuals from each sample were used for microscopic examination of histological smears. A transverse cut was made across the body of the ark shell and a section 3-mm thick was fixed in Bouin's solution. It was then routinely processed for histology and 5-μm paraffin-imbedded sections were stained with iron hematoxylin-eosin (Humason 1979). The stage of gonadal development was classified and scored on a 0 to 4 scale according to a scale of maturity (Ivell 1979). At grade 1, sexes are just identifiable but very little gonad is developed. Grade 2 represents a stage of moderate gonadal development. At this grade, oocytes are beginning to develop. At grade 3, gonads are distended and ripe. At this stage, gametogenesis is complete but spawning has not yet occurred. Oocytes are liberated freely on dissection and free sperm are manifested as streaks. Grade 4 is the spawning stage of animals and in half-spent to completely spent condition. Grade 0 represents both the completely spent stage and the immature stage. At this grade, sexes are unidentifiable. The arithmetic means of the individual scores of the whole sample was recorded as the Gonadal Maturity Index (GMI) for each sampling date (see details in Dinamani 1987).

Biochemical Measurements of Animal Tissue

The dry tissue of 20 individuals was pooled and homogenized to determine average biochemical composition. Protein was determined by the colorimetric method of Lowry et al. (1951) after extraction with normal sodium hydroxyde. Carbohydrate and glycogen were extracted in 15% trichloroacetic acid, and precipitated with 99% ethanol. They were analyzed using the phenol-sulfuric acid method as described by Dubois et al. (1956). Extraction for

total lipid was performed in a mixture of chloroform and methanol (Bligh & Dyer 1959) and lipid content was determined using the method of Marsh and Weinstein (1966). Ash content was obtained by igniting a subsample (30 to 80 mg) of homogenized tissue at 450 C for 48 h in a muffle furnace.

Standard Animal

To evaluate the physiological state of *S. broughtonii* independent of growth, absolute values for tissue weight and biochemical composition of a standard animal of 66.5 mm in shell length were compared for each sampling date. Allometric equations of $\log_{10}$ dry tissue weight against $\log_{10}$ shell length at each sampling date were determined by linear regression analysis. The results of the biochemical analysis were then expressed in milligrams per standard animal. All regressions were statistically significant ($p < 0.01$). A similar method has been used in the studies for the barnacles, *Balanus balanoides* and *B. balnus* (Barnes et al. 1963), and for the tellin, *Tellina tenuis* (Ansell & Trevallion 1967).

RESULTS

Environmental Parameters

Water temperature increased from March to August and decreased from September to February (Fig. 2). The maximum water temperature was in August 2000 (25.8 C) and the minimum in February 2000 (5.3°C). Salinity was generally variable and lower than 30 psu in summer (June to September), and constant and higher than 30 psu in the other period. The maximum salinity was recorded from January to June 2000 (around 33.6 psu) and the minimum in September 1999 (26.7 psu).

Available Food

Seasonal variations in the concentration and composition of SPM in the water column are presented in Figure 3. SPM concentration recorded several peaks throughout the year (Fig. 3a). The concentration of chlorophyll *a* exhibited a clear seasonal pattern characterized by a marked peak in summer when salinity was very low (Fig. 3b). The peak in 1999 was higher (7.5 $\mu g \cdot l^{-1}$) than in 2000 (around 4.0 $\mu g \cdot l^{-1}$), but the former was recorded in September and the latter was during the June to August period. During the rest of the year, the chlorophyll *a* concentration was relatively low. The food material present in SPM, as represented by the sum of protein, carbohydrate and lipid concentrations, showed a marked summer peak (over 400 $\mu g \cdot L^{-1}$) during the phytoplankton bloom of each year, although another peak was recorded in February 2000 (Fig. 3c). Peak values of the food index, calculated as a percentage of the SPM, were related to the phytoplankton bloom (Fig. 3d). Peak value of ca. 4% was also recorded in winter months December 1999 to February 2000. During the last of the year, the food index decreased to values lower than 2%.

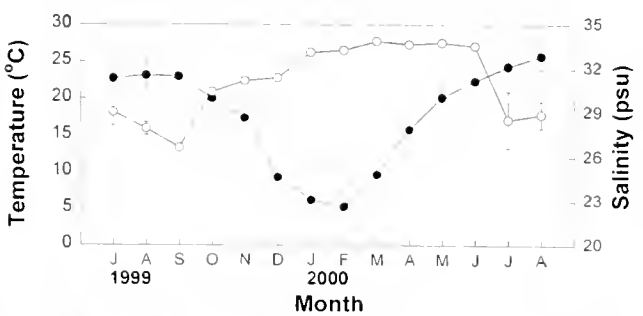

Figure 2. Monthly variations of water temperature (black circles) and salinity (white circles) in Chinju Bay.

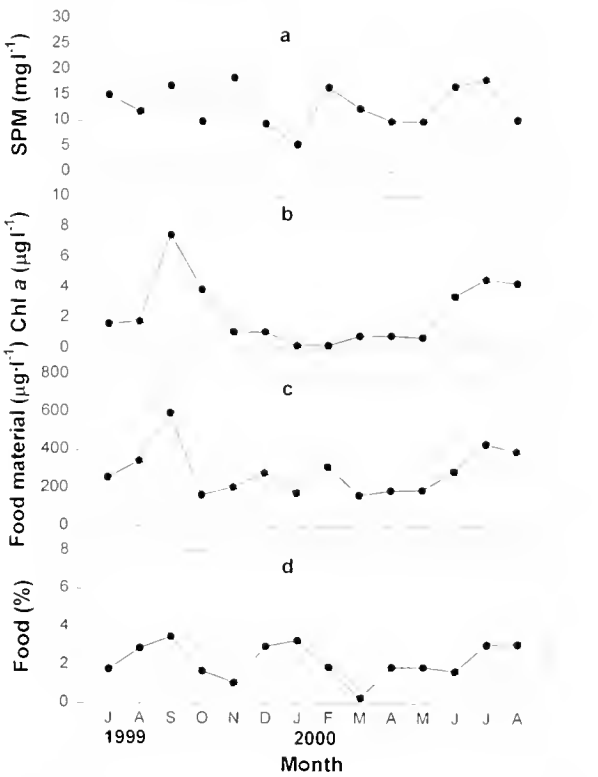

Figure 3. Monthly variations of suspended particulate matter (SPM) (a), chlorophyll *a* (Chl *a*) (b), food material (a sum of particulate protein, carbohydrate and lipid) (c) and food index [(Food material/SPM) × 100] (d).

Condition and Reproductive Cycle

The reproductive cycle of *S. broyghtnii* is characterized by a clear seasonal pattern, as summarized in Figure 4. Maximum values in the condition index were recorded in the early summer months of July 1999 and June 2000, respectively (Fig. 4a). The condition index then decreased sharply to the minimum in late summer of September 1999, suggesting a summer spawning. This index increased progressively from October to June. Since the gametes exhibited the same developmental stage in both female and male follicles, and differences between sexes were not considered in the analysis of biochemical composition, GMI is presented as the means of pooled data from both sexes (Fig. 4b). The development of gonadal tissue started in February and peaked during July to August when the condition index dropped suddenly. Ripe gonads were found from June to August (Fig. 4c). According to the dates of appearance and disappearance of grade 4, which is used as criteria for the beginning and end of spawning, spawning began in July and terminated in September. After spawning, there was a sexual resting period from October to January.

Tissue Weight

Seasonal change in tissue weight for a standard animal (shell length = 66.5 mm) in Chinju Bay is presented in Figure 5. The dry

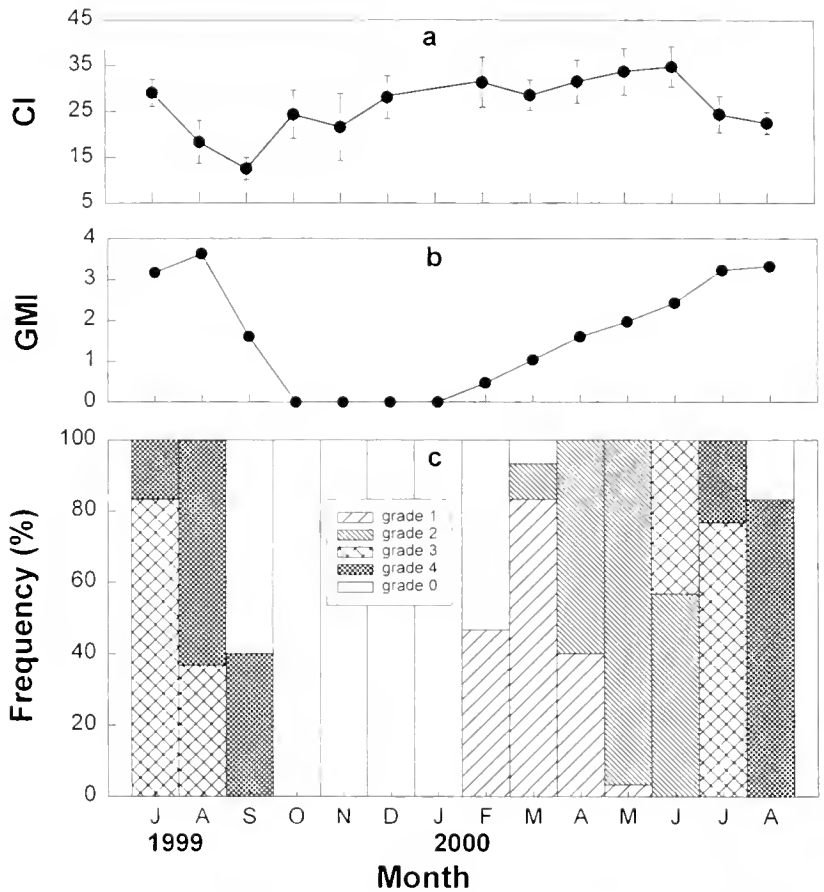

Figure 4. *Scapharca broughtonii.* **Monthly variations of condition (CI) (a), gonad maturity index (GMI) (b) and gonadal development (c).**

tissue weight was maximal in July 1999 and June 2000 (*ca.* 7 mg · standard animal^{-1} for both cases). The tissue weight fell abruptly in summer when spawning occurred. Then, there was a great increase in tissue weight at the timing of sexual resting stage and reserve buildup (see later) during the autumn period (October to December). Thereafter, only a little tissue weight increased steadily during the winter to spring period (February to May).

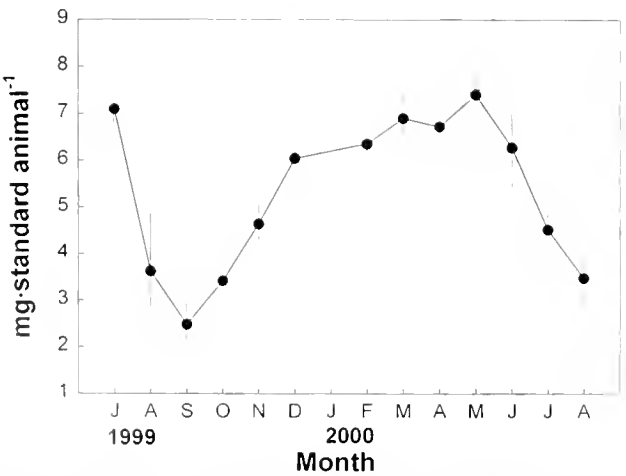

Figure 5. *Scapharca broughtonii.* **Monthly variations of dry tissue weight in a standard animal (shell length = 66.5 mm).**

Gross Biochemical Composition

The gross biochemical composition of a standard animal was calculated from the percentage compositions of each component and the dry tissue weight in Figure 5 and Figure 6 summarizes seasonal cycles of the biochemical components in absolute values as mg dry weight per standard animal. A sudden fall in all the biochemical components of a standard animal occurred during the summer spawning. Thereafter, a great increase in protein and carbohydrate was synchronous with the increase in tissue weight during the autumn period (October to December). At this period, the greater increase in carbohydrate reflected the storage of glycogen reserve in the soft tissue of animals. However, lipid kept lower during this period. During the winter to spring period (February to May), the patterns of variation in each component were different. Protein content continued to increase until May but carbohydrate (also glycogen) content, which was maximal in December, decreased progressively from February to May. Inversely to carbohydrate, lipid content increased steadily during this period. A sharp decrease in all the components was then recorded from June to the end of the study. Ash content showed relatively steady values.

Association between Different Parameters

The Kendall's rank correlation coefficients between the environmental and *Scapharca* measures are presented in Table 1. Chlorophyll *a* concentration was correlated positively ($p < 0.01$) with water temperature but negatively ($p < 0.05$) with salinity, standard

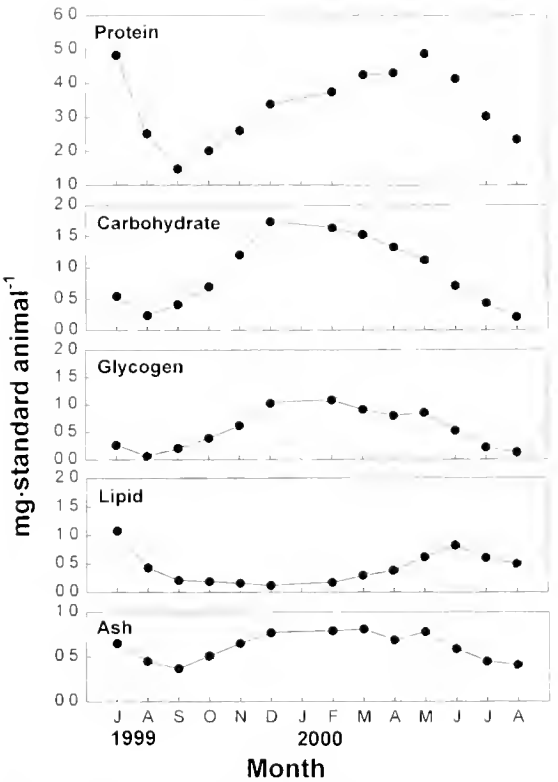

Figure 6. *Scapharca broughtonii*. Monthly variations of biochemical component levels in a standard animal: protein, carbohydrate, glycogen, lipid and ash.

animal protein and carbohydrate (also glycogen) values. Both the condition index and the standard animal dry weight were correlated positively ($p < 0.01$) with its protein value. The GMI correlated positively ($p < 0.01$) with temperature and lipid value but negatively ($p < 0.01$) with carbohydrate (also glycogen) value. With the exception of carbohydrate-glycogen pair, no significant correlation between the absolute values of biochemical components was found.

DISCUSSION

Reproductive Cycle

Changes in temperature, salinity or photoperiod have been considered physical environmental factors, inducing spawning of bivalves (Mann 1979, Dohmen 1985, Lubet et al. 1987). The gametogenesis of *S. broughtonii* in Chinju Bay was characterized by a unimodal cycle, as has been reported for other north Temperate populations of the species in Korea (Park et al. 1998) and Japan coasts (Numaguchi 1996). Positive correlation between GMI and water temperature ($\tau = 0.658$, $p < 0.01$) indicates that temperature might play an important role in inducing the gonadal development and spawning of *S. broughtonii*. A new cycle of gametogenesis began at the lowest water temperature (*ca.* 5.3 C) in February. Similar results were also observed by Park et al. (1998) for other populations of the same species from the southern coast of Korea. However, the lowest water temperature, at which gametogenesis in the latter populations was initiated, was 10°C. This difference indicates that initiation of gametogenesis in *S. broughtonii* occurs at different temperatures in different places, as reported for the oysters *Ostrea edulis* and *Crassostrea gigas* (Ruiz et al. 1992a, b), for the cockle *Cerastoderma edule* (Navarro et al., 1989), for the mussel *Mytilus edulis* (Newell et al. 1982) and for the manila clam *Tapes philippinarum* (Sbrenna and Campioni 1994). In these other species, even spawning also took place at different water temperature. Salinity of Chinju Bay was relatively constant throughout the year with the exception of low salinity in the rainy summer season. No significant correlation between the GMI and salinity was found (Table 1).

Food availability also may have an effect on initiation of spawning. Starr et al. (1990) demonstrated that phytoplankton levels during blooms should be sufficient to induce spawning in sea urchins *Strongylocentrotus drobachiensis* and mussels *M. edulis*. Ruiz et al. (1992a) also postulated that the stimulation by phytoplankton blooms could explain the spawning of *C. gigas* at the low temperature during late October. It is difficult to explicate the summer spawning event by an environmental factor that may trigger spawning. In Chinju Bay, spawning of *S. broughtonii* took place at the moment when water temperature was highest, salinity lowest and photoperiod longest, throughout the year. Also, the blooms of phytoplankton occurred in summer. Kang et al. (1999) reported for the southern coastal bay systems of Korea that the addition of excessive dissolved inorganic nutrients (particularly nitrogen) via streams enhances phytoplankton biomass greatly in the low saline summer period. Therefore, spawning in summer enables larvae to be produced at the most opportune moment with regard to food availability (Newell et al. 1982, see also Navarro et al. 1989).

Numaguchi (1996) observed that spawning of *S. broughtonii* stock cultured in Kasado Bay (Japan) throughout the year took place in insufficient maturation degree from July to September. Spawning of another stock transplanted to Kasado Bay three years after culture in Kagawa Prefecture with the similar latitude and thermal condition was expanded in sufficient maturation from May to October. He concluded that the difference in maturation between two ark shell stocks resulted from nutritive and growth condition. In the present study, the GMI correlated negatively with standard animal carbohydrate and glycogen levels ($\tau = -0.632$ and -0.579, $p < 0.01$ for both). These results indicate that temperature is not the only determinant of gametogenic cycle.

Many authors have taken it into account that nutrient accumulation —depletion cycle in marine bivalves due to variations in ambient food availability may play a critical role in determining their gametogenic cycle. The effect of temperature may be direct by affecting bivalves' metabolic rate, or indirect by affecting the availability of food which increases in summer when temperature is high (Taylor & Venn 1979, Pazos et al. 1997). Both such effects of temperature may be relative to the seasonal cycle of storage and utilization of energy reserves.

Gametogenesis and Biochemical Composition

The gonadal growth in winter to early spring took place at the expense of the stored reserves (mainly glycogen). This was reflected to a negative correlation between the GMI and the glycogen level (Table 1). The gametogenisis in marine bivalves occurs at the expense of the recently ingested food and/or energy stored in various tissues. In the mussel *Mytilus edulis*, glycogen reserves are stored in the mantle (Lubet 1976, Bayne et al. 1982) and are used in the gametogenesis (Gabbott and Bayne 1973). However, in the oyster *Ostrea edulis*, the storage reserves and the gametogenesis take place concurrently (Ruiz et al. 1992b). The relationship between energy storage and gametogenesis within a single species

TABLE 1.

Matrix of Kendall's rank correlation, τ, between the environmental and *Scapharca* measures

	S	SPM	Chl *a*	FM	FI	CI	GMI	DW	P	CHO	G	L	Ash
T	0.410	0.168	0.564**	0.308	0.256	−0.231	0.658**	−0.308	−0.231	−0.872***	−0.821***	0.462*	−0.667**
S		−0.219	−0.538*	−0.385	−0.231	0.513*	−0.211	0.538*	0.513*	0.487*	0.590**	−0.128	0.590**
SPM			0.168	0.194	−0.219	−0.142	0.093	−0.116	−0.142	−0.116	−0.168	0.039	−0.219
Chl *a*				0.282	0.231	−0.410	0.237	−0.590**	−0.513*	−0.590**	−0.641**	0.128	−0.795***
FM					0.590**	−0.256	0.395	−0.333	−0.308	−0.333	−0.282	−0.026	−0.333
FI						−0.205	0.263	−0.333	−0.308	−0.282	−0.231	−0.077	−0.282
CI							0.000	0.615**	0.692**	0.308	0.410	0.308	0.462
GMI								0.000	0.079	−0.632**	−0.579**	0.605	−0.316
DW									0.923***	0.385	0.436*	0.231	0.641**
P										0.308	0.359	0.308	0.564**
CHO											0.897***	−0.385	0.692**
G												−0.282	0.744***
L													−0.128

T = water temperature, S = salinity, SPM = suspended particulate matter, Chl *a* = chlorophyll *a*, FM = food material, FI = % food index, CI = condition index, GMI = gonad maturity index, DW = standard animal dry weight, P = protein, CHO = carbohydrate, G = glycogen, L = lipid). * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$, *** $p < 0.001$.

may depend also upon whether glycogen sufficient to meet the energy requirement for gonadal development, is stored under the feeding condition prior to the gametogenesis. Navarro et al. (1989) reported inter-annual differences in the timing of gametogenesis and the storage cycle of carbohydrate reserves in a population of the cockle *Cerastoderma edule* from Mundaca Estuary (Spain). In fact, gamete proliferation of the cockle population occurred during the expense of glycogen reserves or simultaneously with the accumulation of energy reserves, depending on prior feeding conditions. Such a difference within a species was also observed geographically in the oyster *Crassostrea gigas*. While the glycogen stored is used in the gametogenesis of an oyster population in El Grove (Spain) (Ruiz et al. 1992a), the accumulation of reserve materials and the gametogenesis commenced simultaneously in Korean Waters (Kang et al. 2000). Recently, Luna-González et al. (2000) also found that the scallop *Argopecten ventricosus* uses the available food in the environment more than muscle reserves for the gonadal maturation when the food is abundant, but they use the muscle reserves when the food abundance is poor.

Summer spawning of *S. broughtonii* was followed by a subsequent accumulation period of reserve materials in autumn (from October to December). A great increase in animal tissue weight in autumn was attributed to the rapid accumulation of protein and carbohydrate during that period (Figs. 5 and 6). Glycogen level fell slowly during the course of gonadal development in the winter–spring period. In the present study, the low chlorophyll *a*, food material and food index indicated that both quantity and quality of food were poor during the winter–spring period (from February to May), although a temporary peak in food material was recorded in February (Fig. 3). Therefore, our results indicate clearly that the gametogenesis of *S. broughtonii* in winter–spring depend largely on the glycogen stored during the previous autumn. It is generally accepted that glycogen reserves are the main source of energy in bivalves (Reid 1969, De Zwaan & Zandee 1972) and also may be utilized for the formation of gametes under conditions of nutrient stress (Gabbott and Bayne 1973, Newell and Bayne 1980, Barber and Blake 1981, Beninger and Lucas 1984).

Protein and lipid levels increased steadily during gametogenesis, with maxima prior to spawning. Lipid is converted from glocogen reserves and biosynthesized during the formation of gametes (Gabbott 1975, Lubet 1976). Lipid level may be a good index of gonad maturity (Pazos et al. 1997), because lipid has been considered one of the principal energy sources used during non-feeding embryonic and early larval stages of the bivalves (Holland 1978, Gallager et al. 1986). A positive correlation between the GMI and the lipid level ($\tau = 0.605$, $p < 0.01$) supports these hypotheses. Protein constitutes the major organic component of bivalve oocytes (Holland 1978). Therefore, protein maxima prior to spawning may support this hypothesis. Protein also supports reproduction after carbohydrate and lipid reserves were depleted (Mann & Glomb 1978, Barber & Blake 1981) and constitutes the main reserve during periods of energy imbalance (Gabbott & Bayne 1973, Beninger & Lucas 1984, Navarro et al. 1989). However, the lack of evidence for such roles of protein reserves in the present study may be attributed to the biochemical analyses for pooled tissues. Another possible explanation for the steady increase of protein level in winter–spring is that part of the energy necessary for gametogenesis and maintenance may be supplied by the recently ingested food without intervention of the energy reserves. Analyses for various organs such as striated adductor muscle, digestive gland and gonad will reveal roles of reserve materials in detail, as proposed by Barber and Blake (1981) and Pazos et al. (1997).

In conclusion, *S. broughtonii* from Chinju Bay, a southern coastal bay of Korea, was characterized by a spawning period in summer due to the monocyclic gametogenesis throughout the year. Gametogenesis took place in winter–spring at the expense of reserves accumulated previously in autumn. An inverse relationship between glycogen levels and GMI suggests that carbohydrate played an important role as the gametogenesis fuel. Therefore, this bivalve may be considered a typical conservative species in gametogenic pattern (Bayne 1976).

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A STUDY OF THE ARKSHELL CLAMS, *NOETIA PONDEROSA* (SAY 1822) AND *ANADARA OVALIS* (BRUGUIÈRE 1789), IN THE OCEANSIDE LAGOONS AND TIDAL CREEKS OF VIRGINIA

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ABSTRACT Two species of arkshell ("blood") clams, *Noetia ponderosa* and *Anadara ovalis*, have recently been targeted by watermen on the eastern shore of Virginia for sale to both East and West Coast markets in the United States. Until 1991, fishermen caught both species in the harvest of oysters and hard clams, and discarded them as bycatch with little value. Very little is known about either species of blood clam, and preliminary data from a pilot study in 1993 indicated that they were being over-fished. We conducted a survey in September 1994 in the oceanside lagoon system along the eastern shore of Accomac and Northampton Counties, Virginia, and collected data on density, abundance, habitat preference, age-size and morphometric relationships, and mortality rates for both species of blood clams, as well as some ancillary data on the hard clam, *Mercenaria mercenaria*. The study provides baseline data for establishing management practices and regulations for the blood clam fishery. The total estimated abundance in the study area was about 16 million *N. ponderosa* and 6.4 million *A. ovalis*. Of the clams taken in commercial catches on the oceanside of the eastern shore, *M. mercenaria* constitutes about 84%, *N. ponderosa* 15%, and *A. ovalis* 1%. In our field survey, *M. mercenaria* was the most abundant species (72% of the total catch), followed by *N. ponderosa* (17%) and *A. ovalis* (11%). Densities for blood clams averaged 0.35 clams m^{-2}, or 3,500 clams per hectare, and were highest in shell and shell/mud substrate (1.1 and 1.2 clams m^{-2}, respectively). Growth studies and age-size data show that *A. ovalis* grows about twice as fast as *N. ponderosa* and that market-size *N. ponderosa* (about 56 mm in shell height) may be 8+ years old. We also present information on mortality rates and morphometric relationships for both species of blood clams, and recommendations for maintaining and enhancing the fishery.

KEY WORDS: *Noetia ponderosa, Anadara ovalis*, arkshell, blood clam, growth rate, density, substrate

INTRODUCTION

Since 1991 two species of arkshell or "blood" clams, *Noetia ponderosa* (Say 1822) (ponderous ark) and *Anadara ovalis* (Bruguière 1789) (blood ark), have been harvested by watermen on the eastern shore of Virginia for sale to markets in Washington D.C., New York City, Los Angeles, and Chicago. Long considered a useless incidental catch in the harvest of the hard clam, *Mercenaria mercenaria* (Linnaeus 1758), and the eastern oyster, *Crassostrea virginica* (Gmelin 1791), arkshell clams now constitute a rapidly growing fishery with potential for future development. However, there is very little published information on the life history of either of these species. Chanley (1966) and Chanley and Andrews (1971) described the larval stages of *N. ponderosa* and *Anadara transversa* (Say 1822) in Virginia waters and reported spawning periods of June to December and May through September, respectively. In addition, they stated that *A. transversa* was common in Chesapeake Bay and tributaries with salinities above 15. Loosanoff and Davis (1963) and Loosanoff et al. (1966) also described the larval development of *A. transversa*. McGraw and Castagna (1994) conducted preliminary investigations on growth rates of *A. ovalis* and *N. ponderosa* on the eastern shore of Virginia concurrent with the growth of a blood clam fishery there. Anderson et al. (1985) also reported the potential for a viable blood clam fishery along the South Carolina coast but could not find a market for the clams. Walker (1998) studied the growth and survival of *A. ovalis* in suspended pearl nets in Wassaw Sound, Georgia. The intensive harvesting of blood clams and paucity of data on important factors such as distribution, densities, growth rates, and survival present a problem for management of the fishery in Virginia waters.

Blood clam landings in Virginia (Fig. 1) from 1993 to 1998 (National Oceanic and Atmospheric Administration 2000) show that about 8.9 metric tons of blood clams were harvested in 1993, with a decline to 2.5 metric tons in 1995, and an increase to 10.8 metric tons in 1997. The most logical explanation for the resurgence in tonnage appears to be a change in gear type, from mostly mechanical tongs during the early phase of the fishery to clam dredges after 1996. Landings are reported as wet-meat weights, whereas the clams are usually sold whole, in the shell. Using a conversion factor supplied by the state of Virginia (Iverson pers. comm., Virginia Marine Resources Commission, February, 2000), we estimated that the number of clams range from about 340,000 clams landed in 1995 to 1.5 million clams in 1997.

Most of the blood clams harvested along the eastern shore of Virginia are *N. ponderosa*; however, some *A. ovalis* are included, and buyers make no distinction between the two species. Virginia State fishery regulations concerning the harvest of arkshell clams are currently the same as for hard shell clams, and prohibit dredging from April 1 through December 1. Harvest by mechanical tongs is not regulated by season, and that method is used to continue harvesting during the closed dredging season. Clam fishermen would like to harvest blood clams by dredging year-round to provide consistent supplies to the markets they have developed. They requested a variance from the fisheries regulatory agency, Virginia Marine Resources Commission (VMRC), to permit dredging the arkshell clams during the normally closed season (Terry 1991). However, VMRC denied the request until more information was available on which to base management practices and regulations.

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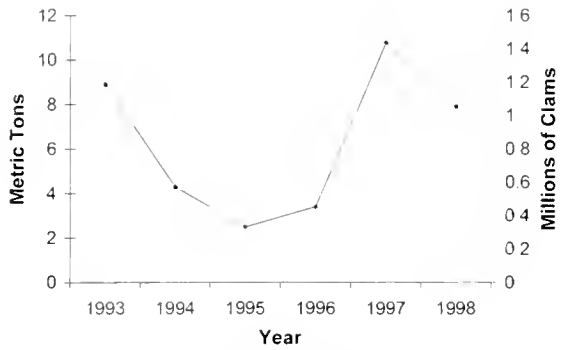

Figure 1. Blood clam landings in Virginia, 1993–1998 (National Oceanic and Atmospheric Administration/National Marine Fisheries Service, http://www.st.nmfs.gov/commercial/index.html).

The number of blood clams sold in retail markets in different regions is difficult to obtain, but two seafood dealers in Washington, D.C. offered some estimates for their stores. One sold 3,000 to 5,000 per week at a price of $2.50 to $3.00 (U.S.) per dozen (Pruitt pers. comm., Washington, D.C., 1993). Another company sold about 2,000 blood clams per week from about November through March for about $3.00 to $4.00 per dozen (Martin pers. comm.,Washington, D.C., 1993). Prices usually range from $0.11 to $0.18 per clam (Bishop pers. comm., Oak Hall, VA, 1996); however, watermen reported receiving from $0.07 to $0.25 per whole clam, depending on the size and demand. One reportedly received $0.50 per clam by selling the clams directly from his boat (Annis pers. comm., Willis Wharf, VA, 1993).

Blood clams from Virginia are sold primarily in ethnic markets in the U.S. and are eaten raw and cooked. Both species have a somewhat bitter taste and contain the blood pigment hemoglobin, which gives the flesh a blood-red color (Abbott 1968, Yonge and Thompson 1976). These attributes may explain why they are not usually eaten in the U.S.; however, various ark species (also called blood cockles in some countries) constitute significant fisheries in many other parts of the world, including China, West Africa, Japan, Mexico, Tanzania, India, Thailand, Malasia, and Taiwan (Bae 1986, Baqueiro 1980, Broom 1983, Broom 1985, Guo et al. 1999, Ismail 1986, Kayombo 1993, Narasimham 1969, Narasimham 1988, Nie 1990, Sahavacharin et al. 1988, Ting 1984, Wong and Lim 1985). Prior to 1950 there were also substantial harvests of *Arca noae* (up to 685 tons per year) from the Adriatic Sea (Hrs-Brenko 1980).

The ponderous ark (*N. ponderosa*) has a heavy, thick shell, is ubiquitous along the oceanside of the eastern shore of Virginia, and aggregates in shell debris or "shell hash" where juveniles attach by a prominent byssus to whole shells and pieces of shell (McGraw and Castagna 1994, McGraw et al. 1996). This species ranges from Virginia to the Florida Keys and in the Gulf of Mexico from Key West to Texas. Virginia is thought to be the northernmost extension of the range, and shells found north of Virginia are probably fossils (Abbott 1954). Because they have no siphons, as some other clams do, ponderous arks are usually found at the substrate surface, making them very accessible to dredges and tongs. Market sized *N. ponderosa* may include animals over ten years old (McGraw et al. 1996).

The blood ark (*Anadara ovalis*) is found from Cape Cod to the Gulf of Mexico, the Caribbean, and West Indies (Abbott 1954, Gosner 1978) and occurs subtidally from shallow water to 45 m both in shell and muddy substrates. It has a much thinner shell than the ponderous ark. In salinity tolerance studies, Castagna and Chanley (1973) found that *A. ovalis* and *N. ponderosa* generally functioned better in salinities exceeding 20.

The primary purpose of our study was to gather data on the growth rate, size-frequencies, ages, densities, abundance, and survival of blood clams on the eastern shore, and to make these data available to fisheries management agencies for consideration in overseeing the fishery. Unlimited harvesting, coupled with the slow growth rate of *N. ponderosa* and insufficient recruitment, could eventually lead to depletion of blood clam populations if present harvest practices persist.

METHODOLOGY

The study consisted of four main parts: (1) conducting a field survey to obtain data on density, substrate preference, distribution, abundance, and mortality rates for the two species of blood clams in the tidal lagoons of the eastern shore of Virginia; (2) determining growth rates from age-size relationships using the acetate peel technique; (3) collecting fisheries catch data from local watermen; and (4) following the growth rates of cohorts of *A. ovalis* and *N. ponderosa* over a 2-y period. Although the primary emphasis of the study was on blood clams, we collected some data for the hard clam (*Mercenaria mercenaria*), and some of that information is also included for comparison, since it is the primary species harvested.

Field Survey

We conducted a field survey aboard a commercial fishing vessel rigged with mechanical tongs during September 1994. The main sampling area (Fig. 2) was the oceanside of the eastern shore of Virginia from the Great Machipongo Inlet to Wachapreague Inlet, or the general area between longitudes 75°42′–50′W and latitudes 37°20′–38′N. We employed both random and systematic sampling techniques, depending on the area being sampled. In Hog Island Bay, depth of water permitted more random sampling, both in channels and over mud flats, whereas the more northern part of the study area was better suited to systematic sampling in channels.

We determined random sampling sites by setting up a grid overlay (800 m × 800 m blocks) of the Hog Island Bay area (NOAA chart #1221) and used random number tables to determine which blocks we would sample. We took three samples within each chosen block, and the sampling locations within the block

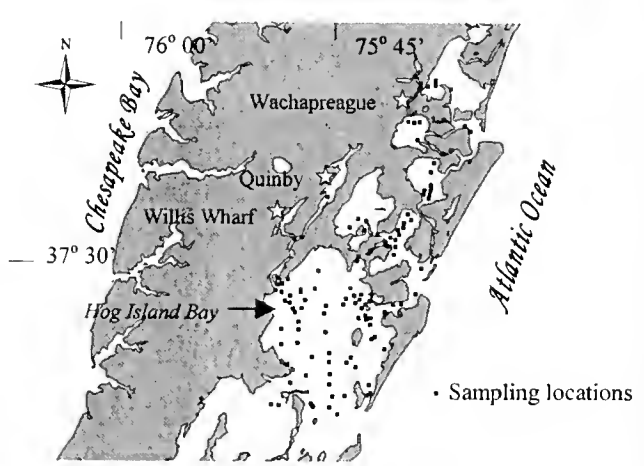

Figure 2. Map of study area.

constituted one station. If water depth in a block was too shallow, we eliminated the sampling site and selected additional random numbers until another suitable block was chosen.

In addition to the blocks chosen by random numbers, we included portions of some of the following tidal creeks and channels in a systematic sampling plan: Swash Creek, Sandy Creek, Sloop Channel, Parting Creek, Machipongo River, Great Machipongo Channel, Quinby Inlet, Sand Island Channel, Millstone Creek, and Wachapreague Inlet. In tidal creeks and channels we took three samples approximately every 0.5 miles or 900 m, usually in the immediate vicinity of channel markers to more precisely locate positions on navigation charts. Channel samples usually spanned the channel or creek along transects, with one sample from the middle and one from each side. We sampled a total of 119 stations.

We used mechanical tongs for sampling gear instead of a dredge because tongs cover a discrete area, 1.12 m^2, penetrate into the substrate about 15 cm, and retain more substrate when retrieved from the bottom. Retention of substrate and small clams was assured by lining the tongs with 1 cm^2 plastic mesh. Area is a more pertinent measurement than volume for assessing densities of blood clams because the clams inhabit the upper 6–8 cm of substrate and are easily caught with tongs. We placed samples in plastic bags or buckets and sorted them on shore.

We processed samples on shore by washing them through 1 cm^2 mesh screens (corresponding to the mesh size lining the tongs) and counted and measured all clams (height, length, and depth) to the nearest mm with vernier calipers. Height is defined as the distance between the dorsal hinge (umbo) and the ventral lip of the clam (Fig. 3), and length is the distance from the anterior end to the posterior end. Depth or thickness is the greatest distance between the right and left valves. We used morphometric data to construct size-frequency distributions for both species of blood clams as well as to determine relationships between height, length, and depth for *N. ponderosa* and *A. ovalis*. We also weighed some clams to determine the correlation between height, whole weight, and wet meat weight.

Our preliminary observations (McGraw and Castagna 1994) indicated that *N. ponderosa* is found almost exclusively in areas with shell and shell debris. Because random survey samples contained varying substrates, we could evaluate habitat preferences for *N. ponderosa* and *A. ovalis*. We qualitatively placed substrate material in each sample into the categories used by Haven et al. (1981) (i.e., mud, sand, shell, shell/mud, shell/sand, and sand/mud) and provided areal estimates of each. Space limitations of the vessel and the volume of substrate in some samples dictated that only portions of samples (i.e., sub-samples) be retained for sorting. In those instances, we noted sub-sample proportions, along with other data, and factored those into the density calculations during data analyses to obtain adjusted densities. We estimated densities for each species at each station based on the number of clams obtained per sample and surface area covered by the tongs (i.e., approximately 1.12 m^2).

Analysis of field data showed that the general distribution of clams was clustered or aggregated (i.e., non-normally distributed), as evidenced by coefficients of dispersion > 1 (Sokal and Rohlf, 1969). Therefore, we transformed density data using log (X + 1) transformation according to the method discussed in Zar (1974) and Sokal and Rohlf (1969) before we applied analysis of variance (ANOVA) or other statistical tests (e.g., to test for differences between mean densities among substrate types). After transformation, we calculated clam densities by dividing the number of clams caught in each sample by the area covered by the mechanical tongs (i.e., 1.12 m^2). Then we tested mean densities of clams (both species combined) found in the various substrates using ANOVA (Sokal and Rohlf 1969) and the Student-Newman-Keuls (SNK) test (Zar 1974) to determine which means, if any, were significantly different ($\alpha = 0.05$). We analyzed data further according to substrate type and water depth (i.e., deep water/channels or mud flats/shallow areas).

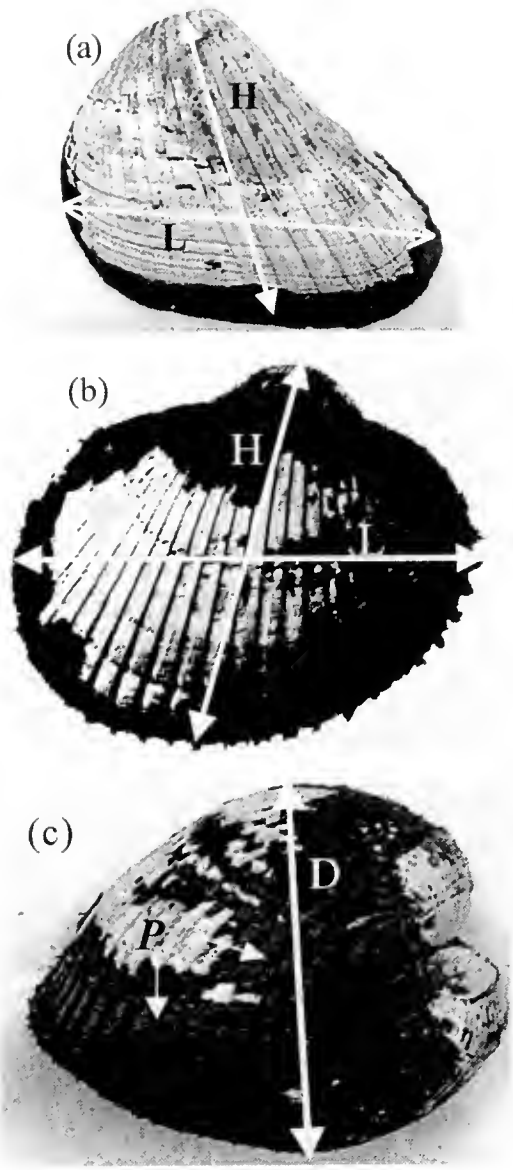

Figure 3. ***Noetia ponderosa*** **(a) and** ***Anadara ovalis*** **(b) showing length (L) and height (H) dimensions; (c)** ***Anadara ovalis*** **showing depth (D) dimension and periostracum layer (*P*).**

Where substrate type was the same for two or three samples from a station, we averaged those for each station; if substrate types differed, we treated them as separate samples. Therefore the number of samples for comparing species by substrate types was 169 instead of 119.

Shell Aging

We used acetate peels on different sizes of blood clams to determine age more precisely and to estimate the maximum lon-

gevity of the clams. The technique has long been used by paleontologists (Rigby and Clark 1965), but has proven effective in age determination for several species of bivalves (Ropes and O'Brien 1979, Ropes 1984, Ropes 1987, Kennish 1980). We specifically employed Farrow's (1971) method, which eliminates the step of embedding shells in epoxy resin. In addition, we modified Farrow's (1971) method by using thick (1.56 mm) acetate and cutting it into 7.6 cm × 2.54 cm pieces (i.e., the size of microscope slides) that could fit easily onto the mechanical stage of a compound microscope.

Several authors have discussed shell microgrowth patterns in detail, including Pannella and MacClintock (1968), Rhoads and Pannella (1970), and Lutz and Rhoads (1980). Age and size data can be applied to size distribution data through back-calculation procedures to create age frequency distributions, thus providing a better understanding of the population structure (Robson and Chapman 1961, Gulland 1966, Ricker 1975). We determined ages for all blood clams from the field survey and increased sample size and accuracy by supplementing these with data from previous studies and additional clams purchased from local watermen. Then we constructed Von Bertalanffy growth curves (Ricker 1975) for both species of blood clams.

Mortality Rates

We used articulated clam shells from survey samples to estimate annual age-specific and instantaneous mortality rates for both species. Some bivalves remain articulated for a time after death before the hinge ligament deteriorates and the valves separate. These can be used to help estimate natural mortality (Dickie 1955, Buckner 1984) by dividing the number of articulated shells by the sum of live clams plus articulated clams for different size/age groups. There are no data documenting the length of time for deterioration of the hinge ligament for either *N. ponderosa* or *A. ovalis* in Virginia waters, and all mortality rate calculations are based on an assumed time period of one year for disarticulation. We computed instantaneous mortality rates using the equation $(Z) = -\log E (1 - A)$, where A = the number of living clams in an age group and E = 2.718, the base of natural logarithms (Ricker 1975). First, we applied age-length data from acetate peels to the size distribution of living and articulated clams from the survey and determined the number of clams in each age-size class. Then we divided the number of articulated shells in a given age-size category by the number of live clams to arrive at an age specific or annual mortality rate expressed as a percentage.

Commercial Catch Data

Mr. David Bishop (Oakhall, VA, Accomac County) collected and recorded data on the proportion of blood clams versus hard clams from many of his tong catches over a three-month period, from September through November, 1994. We used height-frequency data from his catches for comparison with data from 1993 fisheries samples to help determine if average clam size in commercial catches was decreasing and also to more accurately assess the percentage of blood clams in clam harvests.

RESULTS AND DISCUSSION

Field Survey Results

Clam Density and Abundance

We took a total of 355 individual samples at 119 stations, which yielded adjusted totals of 86 *N. ponderosa*, and 55 *A. ovalis*. The 119 stations included random stations, mostly in Hog Island Bay, as well as non-random, or systematic stations, in the northern part of the study area, taken mostly in channels or creeks. Before combining data from all stations, we first ascertained (using transformed data and ANOVA) that there was no significant difference ($\alpha = 0.05$) in mean clam densities (total densities or by species) between random and non-random stations.

For the non-random (mostly channel) stations, we also tested to see if there was any correlation between clam densities and station location within particular waterways, as defined by distance (nautical miles) from the entrance of a channel (Scheaffer et al. 1996). We found no correlation or trends (maximum $r^2 = 0.06$ in the Great Machipongo Channel) and combined all non-random channel stations for further analyses.

Average density (mean ± standard error = $\bar{X}$ ± S.E.) at all stations ($n = 119$) was 0.21 ± 0.06 clams m^{-2} for *N. ponderosa* and 0.14 ± 0.07 clams m^{-2} for *A. ovalis*. Total average blood clam density was 0.35 ± 0.10 clams m^{-2}. Compared to hard clams, 17% of the total catch was *N. ponderosa* and 11% was *A. ovalis*.

To compare densities by substrate, we averaged densities for each substrate at each station; if station samples contained two or more different substrates, those were considered separate samples. We did this to minimize any pseudoreplication (Hurlbert 1984) and to avoid combining samples across substrates. Therefore, sample size for comparing substrates was 169 (i.e., at some stations not all samples were of the same substrate). In addition, because the mean densities are weighted differently when categorized by substrate type, they are slightly different from those calculated for stations, where the sample size was 119. The proportion of different substrate types among stations was as follows: mud = 35%, $n = 59$; sand = 11%, $n = 18$; sand/mud = 21%, $n = 36$; shell/mud = 17%, $n = 29$; shell/sand = 6%, $n = 11$; and shell = 10%, $n = 16$.

Mean clam densities varied among substrate types (Fig. 4) and were highest in shell (1.24 ± 0.4 clams m^{-2}) and shell/mud substrates (1.12 ± 0.49 clams m^{-2}). *Noetia ponderosa* accounted for most or all clam densities in those two substrates. For example, mean density of *N. ponderosa* was highest in shell/mud substrate (1.12 clams m^{-2} ± 0.49) and shell (0.79 ± 0.36), whereas *A. ovalis* densities were highest in shell substrate (0.45 ± 0.24 clams m^{-2}) and mud substrate (0.19 ± 0.15). The highest density of *N. ponderosa* we observed in a single sample was 13.4 clams m^{-2} at a station in the Great Machipongo Channel (intracoastal waterway); the mean density at that station was 4.46 clams m^{-2}. The highest

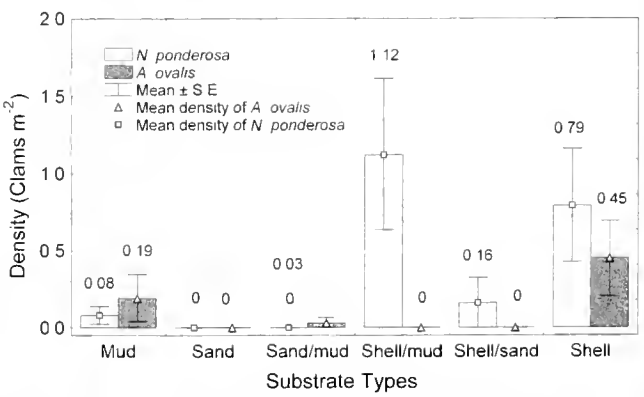

Figure 4. Comparison of *A. ovalis* and *N. ponderosa* densities (clams m^{-2}) in various substrates from the eastern shore of Virginia.

density of *A. ovalis* was 15.0 clams m^{-2} from a sample in The Deeps Channel, a branch of the Great Machipongo Channel.

After transforming density (i.e., log [X + 1]), we compared mean blood clam densities for the six substrate types using ANOVA ($\alpha = 0.05$) and the Student-Newman-Keuls test (Zar 1974) to determine which, if any, means were different. There was a significant difference in mean total blood clam densities among substrates ($P < 0.001$). There were no significant differences in mean total blood clam densities between shell and shell/mud substrates, or between mud substrate and shell/sand, sand/mud, or sand (Table 1 and Fig. 4). However, there were significant differences in mean densities between both shell or shell/mud substrates and all other substrate types ($P < 0.001$). Results are summarized as follows: (shell = shell/mud) ≠ (mud = shell/sand = sand/mud = sand).

Next, we compared densities for both species by substrate type. Results were the same for analyses of *N. ponderosa* densities by substrate types as for both species combined. However, density of *A. ovalis* in shell substrate was significantly different from those in all other substrates, i.e., shell ≠ (shell/mud = mud = shell/sand = sand/mud = sand). The higher densities of clams in shell and shell/mud substrates suggests that shell is important either for attachment, protection from predation, or both.

We estimated the abundance of clams (Table 1) by using substrate data from Haven et al. (1981) and multiplying the density of clams found in various substrates by the number of hectares of that substrate for the study area. That is, $A_t = \Sigma$ (Ds × ha), where A_t = the total abundance of clams in the study area; Ds = the total mean density of clams in a given type of substrate; and ha = number of hectares of a given substrate in the study area. Haven et al. (1981) estimated the following amounts (converted to hectares here): shell = 116 ha; shell/sand = 838 ha; shell/mud = 1,002 ha; sand = 1,523 ha; mud = 2,933 ha; sand/mud = 1,057 ha. The combined areal totals from Haven et al. (1981) are for Burton's Bay, Bradford Bay, Swash Bay, Upshur Bay, Major Hole Bay, Revel Island Bay, Hog Island Bay (above and below North Channel), Ramshorn Bay, and Sand Shoal Channel. Total estimated blood clam abundance in the study area (Hog Island Bay, Burton's Bay, and Bradford Bay and contiguous waterways) was 22 million blood clams. Total estimated abundance by species is as follows: *N. ponderosa*, about 16 million; *A. ovalis*, about 6 million. The proportions are based on those from the field survey (Table 1) in which *N. ponderosa* had an average density of 0.31 clams m^{-2} ± 0.09 ($n = 169$), and *A. ovalis* of 0.12 clams m^{-2} ± 0.06 ($n = 169$) over all substrates.

We examined clam densities in relation to water depth (i.e., channels or mudflats). Of the 119 stations sampled, 67 were in channels or locations with a water depth >2 meters, and 52 were over mudflats, or in shallower water. Using log transformation and Student's *t*-test (Zar 1974), we determined that there was no significant difference ($\alpha = 0.05$) in mean total blood clam densities (Fig. 5) between channel/deep water stations (0.48 ± 1.3) and mud flat stations (0.19 ± 0.10), even though channel stations had twice the density of shallower ones. The relatively high variances for both means affected results and $P = 0.052$, just slightly more than the stated level of significance.

Although mean clam densities were higher at channel/deep water stations, there were no significant differences ($P > 0.10$) between mean densities in channel and mud flat stations for either species (Fig. 5). One explanation for the higher density of clams in the channels/deep water stations might be that clams are sloughed off or eroded from the sides of the channels, along with substrate, and aggregate in the bottom of the channel. In some of the tidal creeks currents may expose areas of shells, providing more attachment sites for blood clams, particularly *N. ponderosa*.

Size-Frequency

Average shell height for blood clam species (Fig. 6) were: *N. ponderosa*, 42.6 mm (± 2.2, $n = 43$) and *A. ovalis*, 25.1 mm (± 0.2, $n = 29$). There were relatively few small *N. ponderosa* (i.e., <25 mm, or about 2 years old) taken in survey samples. This could simply be the result of sampling variability, but could also indicate that recruitment may be low or that mortality rates may be high during the first year after settlement. Most of the hard clams (*M. mercenaria*) in samples were also larger, 60 to 100 mm in height (75.3 ± 1.2, $n = 146$), and sizes were more normally distributed (Fig. 6c). In contrast, most of the *A. ovalis* (Fig. 6a) were 0+ to 2 years old, with very few older, larger clams in samples.

Articulated Shells and Mortality

After calculating Von Bertalanffy growth curves for both species of blood clams, we applied age-length data to articulated shells, put them into age categories, and estimated annual and instantaneous mortality rates (Table 2) as previously described. In the absence of published or other data on the length of time for

TABLE 1.

Mean densities (m^{-2}) of clams (±S.E.) by species and substrate types, areal estimates of substrate types (from Haven 1981), and clam abundances.

	Substrate type						
Species	Shell (N = 16)	Shell/Sand (N = 11)	Shell/Mud (N = 29)	Sand (N = 18)	Mud (N = 59)	Sand/Mud (N = 36)	Total avg. density (N = 169)
N. ponderosa	0.79 (±0.36)	0.16 (±0.16)	1.12 (±0.49)	0	0.08 (±0.06)	0	0.31 (±0.09)
A. ovalis	0.45 (±0.24)	0	0	0	0.19 (±0.15)	0.03 (±0.03)	0.12 (±0.06)
Total	1.24 (±0.45)	0.16 (±0.16)	1.12 (±0.49)	0	0.27 (±0.15)	0.03 (±0.03)	0.42 (±1.49)
Hectares	116	838	1,002	1,524	2,934	1,057	7,470
Clam abundance (millions)	1.4	1.3	11.2	0	7.9	0.3	22.2

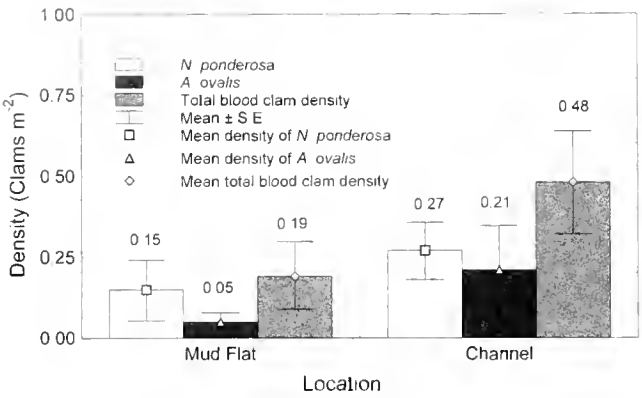

Figure 5. Comparison of blood clam densities (clams m^{-2}) on mudflats and in channels from the eastern shore of Virginia.

disarticulation of shells for either species of blood clam, we assumed a period of one year. Because blood clam density in the study area was relatively low, there were some age groups for which no articulated clams appeared in samples and thus mortality rates were zero. Despite the gaps in data, we think that the mortality estimates provide some insight into basic mortality trends for *N. ponderosa* and *A. ovalis*.

The annual mortality rate (Fig. 7) for 0+ to 1 year *A. ovalis*, calculated using articulated shells in samples, is 86%, then decreases to 30% for the 1+ to 2 year class. Because there were only four articulated clams in the age 3+ category, all less than 48 mm, we pooled the data for a better estimate of mortality rates. The average was 80% for age 3+ clams. Distributed over the estimated maximum life span of six years for *A. ovalis*, the annual mortality rate would be about 27% per year for clams over age 3+. There were no articulated shells in the 2+ to 3 year size range in our samples, so the annual mortality rate for that year class of *A. ovalis* was zero. The increase in mortality rates for older (>3+) clams may be due to senescence or other factors such as disease, or synergistic effects involving spawning and higher water temperatures in the summer. Toyo et al. (1978) (as cited by Broom 1985) reported a sudden mass mortality of a species of *Anadara* (probably *A. broughtoni*) in Japan due to a rapid rise in water temperature above 25°C.

TABLE 2.

Mortality rates (%) and instantaneous mortality rates (Ẑ) for *A. ovalis* and *N. ponderosa*, based on articulated clam shells.

Year class	No. articulated shells	No. live	Total	Mortality (%)	(Ẑ)
A. ovalis					
0–1	70	11	81	86.4	2.0
1+–2	7	16	23	30.4	0.4
2+–3	0	1	1	0.0	0.0
>3+[a]	4	1	5	80.0	1.6
N. ponderosa					
0–1	8	1	9	88.9	2.2
1+–2	3	2	5	60.0	0.9
2+–3	0	9	9	0.0	0
3+–4	0	3	3	0.0	0
4+–5	0	3	3	0.0	0
5+–10[b]	6	11	17	35.3	0.1
10+–15[c]	5	14	19	26.3	0.1

[a] Pooled because of low number of clams in age category. Mortality is estimated to be about 27% per year and (Ẑ) is estimated to be about 0.54 per year for age 3 +– 6 clams.

[b] Mortality is estimated to be about 7% per year and (Ẑ) is estimated to be about 0.1 per year for age 5 +– 10 clams.

[c] Mortality is estimated to be about 5.3% per year and (Ẑ) is estimated to be about 0.1 per year for age 10 +– 15 clams.

Although our estimates are based on relatively few articulated clams, we feel that the data reflect the actual situation, because very few large *A. ovalis* (>50 mm in height) are taken in commercial clam catches and most seem to die before reaching six years of age. A similar phenomenon has been observed for the bay scallop, *Argopecten irradians*, in which about 80% die between months 13–16 (Castagna 1975, Castagna and Duggan 1971). Observations of large *A. ovalis* (i.e., ~53 mm in height) held in flowing water tables indicate that they are sensitive to water temperatures and begin to die above about 27°C, whereas large *N. ponderosa* in the same water tables are not affected. Some watermen have told us that some *A. ovalis* in their catches seem to gape much more readily than *N. ponderosa* during warm weather. It appears that few *A. ovalis* live longer than six years in the Virginia lagoon system, and that mortalities of older clams are exacerbated by high water temperatures.

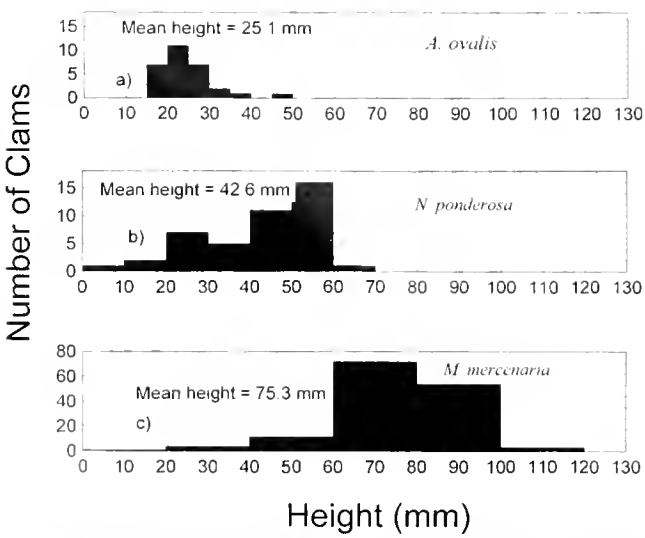

Figure 6. Height frequency distribution for *A. ovalis*, *N. ponderosa*, and *M. mercenaria*.

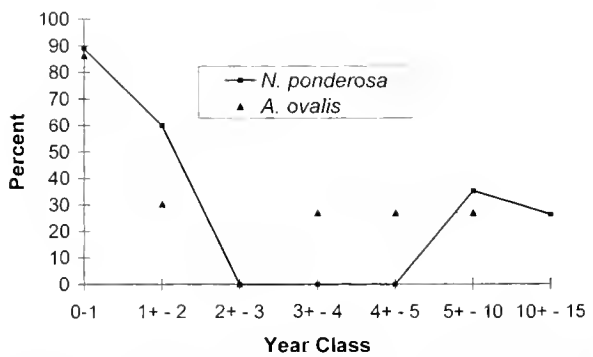

Figure 7. Comparison of mortality rates for *N. ponderosa* and *A. ovalis*.

Walker (1998) reported mortality rates of about 55% for small and medium (<20 mm in length) *A. ovalis* held in pearl nets in Georgia (i.e., from an initial group of 78 clams down to 35) during the first year; average mortality rate for the same groups was about the same during the second year of growth (i.e., 16 out of 35 clams survived). Presumably the pearl nets decreased mortality rates from siltation and predation, and therefore rates were lower than those calculated in our field study. Walker (1998) also stated that the life span for *A. ovalis* in Georgia waters is about three years, whereas we estimated that the life span is six years in Virginia.

Annual mortality rates for *N. ponderosa* (Table 2 and Fig. 7) were highest for the 0+ to 2-year-old groups (89% and 60%, respectively). For convenience sake, and because of small sample sizes, we grouped the 5+ to 10-year-classes and 10+ to 15-year-old clams to determine mortality rates. The cumulative annual mortality rate for the 5+ to 10-year-old group was 35% over the five year period, or about 7% per year; for the 10+ to 15-year-old clams, it was 26%, or about 5% per year.

There were no articulated *N. ponderosa* for the 2+ to 5-y age classes in samples, suggesting a decreasing mortality rate for those age classes. This trend could be a result of increasing size and lower densities during the first two years, as a function of competition for food and space.

Morphometrics

The data used for morphometric relationships are from several sources, including growth studies, fisheries samples, survey samples, and extra clams purchased for shell aging studies. We examined the relationships of several variables: shell height, length, depth, whole weight, and wet weight.

The relationship between valve height and length for *N. ponderosa* (Fig. 8a) is described by the regression equation: $L = 1.22H + 1.73$ ($r^2 = 0.99$) where L = length in mm, H = height in mm, and r^2 = coefficient of determination. Height and shell depth were also linearly related (Fig. 8b) as described by the regression equation $D = 0.98H - 2.57$ ($r^2 = 0.99$), where D = depth of the clam in mm.

Relationships between height and whole (i.e., shell + meat) or wet meat weights were nonlinear (Figs. 8c and 8d). For example, the relationship of height and whole weight is described by the allometric equation of the form $W = aH^b$ (Fig. 8c) where W = whole weight of the clam in grams, H = height in mm, and a and b are allometric coefficients (a = 0.0006 and b = 3.06). Transformed to the linear form, this equation is: $\log W = b\log H + \log a$. Wet meat weight and height were likewise nonlinearly related by the equation $M = aH^b$, where M = meat weight, $a = 0.001$, and $b = 2.52$ (Fig. 8d). The coefficient of determination, $r^2 = 0.88$, is slightly lower than that for height and whole weight, since meat weight determinations are subject to more sampling error, mostly because of varying amounts of water loss. Mean shell weight was 46.8 g (± 36.3, $n = 132$), and mean whole wet weight of *N. ponderosa* was 60.5 g (± 44.06, $n = 132$), or about 77% of the total weight. Mean meat weight (13.6 g) was about 23% of the total weight of the clams sampled.

Relationships of shell height to length or depth for *A. ovalis* were linear (Figs. 9a and 9b). Unlike *N. ponderosa*, shell length in *A. ovalis* changes little in relationship to the height, and most clams are, as the name ("*ovalis*") implies, oval or nearly round. By comparison, increase in shell depth in *A. ovalis* per increase in shell height is proportionately smaller than that in *N. ponderosa*, in which shell depth is almost equal that of height. For *A. ovalis*, shell depth is about 70% of shell height.

The relationship of whole weight and height in *A. ovalis* (Fig. 9c) is best described by the curvilinear equation: $W = aH^b$, where W = whole weight in grams, $a = 0.0003$, and $b = 3.14$ ($r^2 = 0.97$). As with *N. ponderosa*, the correlation ($M = 4E^{-05}\ H^{3.33}$) between height and wet meat weight (Fig. 9d) was more variable than with whole weight, and the coefficient of determination was slightly lower ($r^2 = 0.89$) than for the regression of height and whole weight. Mean whole weight for the *A. ovalis* sample was 34.2 g (± 21.2, $n = 139$), mean shell weight was 22.7 g (± 14.2,

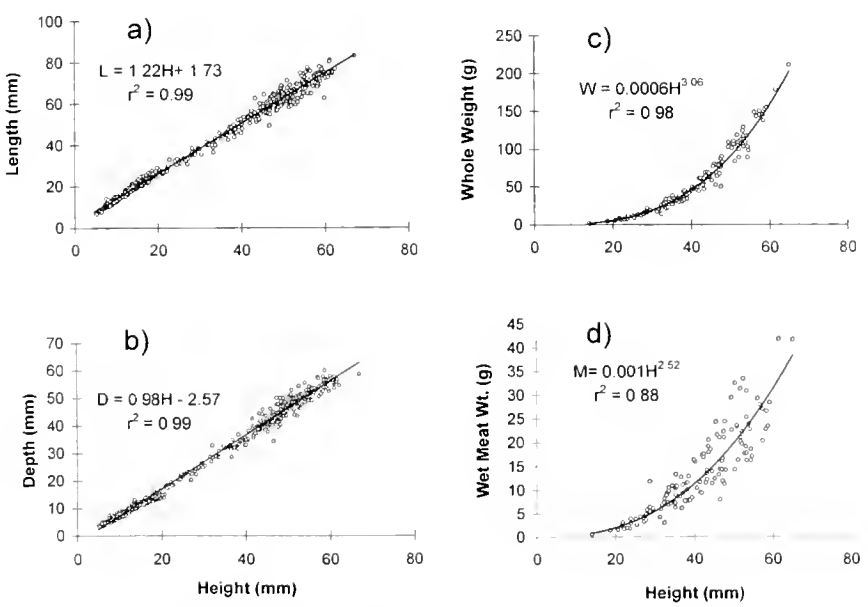

Figure 8. Regression of height versus length (a), depth (b), whole weight (c), and wet meat weight (d) for *N. ponderosa*. Data are from field surveys, commercial fisheries samples, and growth studies ($n = 540$ for a and b; $n = 132$ for c and d).

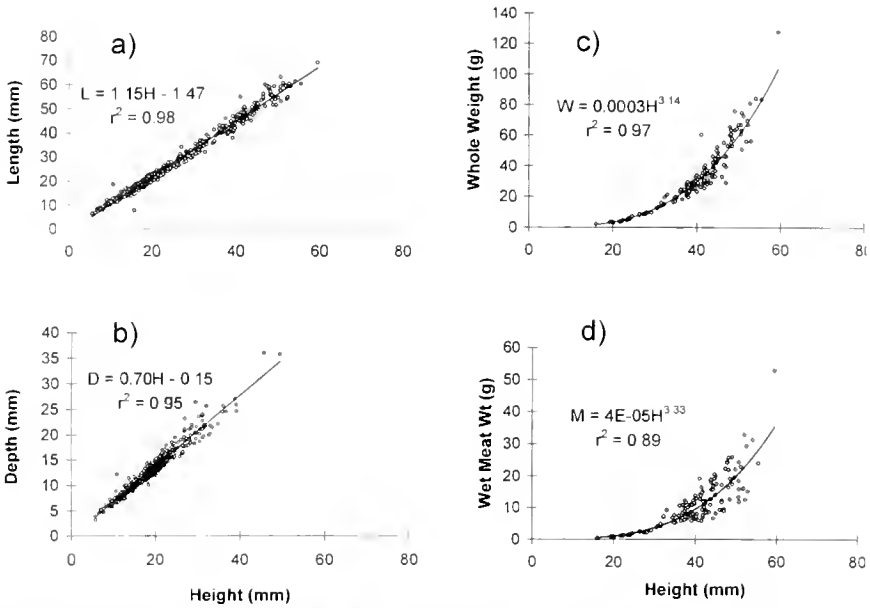

Figure 9. Regression of height versus length (a), depth (b), whole weight (c) and wet meat weight (d) for *A. ovalis*. Data are from field surveys, commercial fisheries samples, and growth studies (n = 778 for a; n = 641 for b; n = 139 for c and d).

n = 139), and mean wet meat weight was 11.5 g (± 8.2, n = 139). Therefore, shell weight in *A. ovalis* constituted about 66% of total weight, and meat weight 34%, or an average of about 10% more meat weight for a given size than in *N. ponderosa*.

Age-size Relationships

We prepared acetate peels from clams taken in the field survey, augmented with clams purchased from fishermen. Some data from growth studies (1992 to 1994) were incorporated as baseline data points for 1- and 2-y-old *A. ovalis* and *N. ponderosa*. We fit age-length data to the Von Bertalanffy growth equation:

$$L_{(t)} = L_{\infty} (1 - e^{-kt})$$

where $L_{(t)}$ is length at time t; L_{∞} is the asymptotic, or maximum theoretical length; and K is a growth constant indicating the rate at which L_{∞} is approached. The L_{∞} and K estimates for *N. ponderosa* (Fig. 10a) are: L_{∞} = 71.5 mm and K = 0.24 (r^2 = 0.94). Both of these parameters are very similar to those given by Cahn (1951) for *A. granosa bisenensis* in Japan.

Mean length of *N. ponderosa* from a commercial fishery sample in 1992 was about 70 mm. Our data indicate that clams >70 mm in length would be 10+ years old. Samples of commercial catches (taken in 1994) from the same vicinity (Parting Creek) showed that the average length of *N. ponderosa* was about 56 mm, a decrease of about 14 mm, or the size of clams of about age 5+. In addition, mean length for *N. ponderosa* in field survey samples was about 54 mm, which also indicates that the older clams are being depleted, and smaller, younger clams are now being harvested. It is possible that some of the clams harvested are older, stunted clams; however, the size ranges for different age groups in the growth model suggest that this is not the case.

The Von Bertalanffy model also provides a good fit for age and size data for *A. ovalis* (Fig. 10b). Values for L_{∞} and K are 57.5 and 0.45, respectively (r^2 = 0.83). Mean length for *A. ovalis* taken in the field survey was 27 mm, or about 1+ year-class clams, while mean length for *A. ovalis* in 1994 commercial fisheries samples was 56.5 mm, or 5+ years old. We obtained only five *A. ovalis* over five years in age in survey samples, which suggests that this species does not live very long in this geographic area. This is also corroborated by the mortality data from articulated *A. ovalis* shells, and from laboratory observations where large *A. ovalis* held in water tables died as ambient water temperatures approached 27°C.

Commercial Fisheries Catch Data

Catch data from a commercial fisherman on the eastern shore (D. Bishop, unpubl.) showed that *M. mercenaria* constituted an

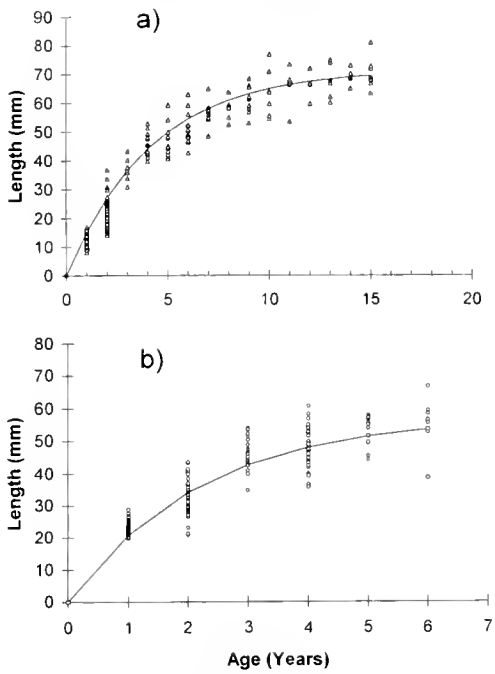

Figure 10. Von Bertalanffy growth curve for *N. ponderosa* (a), n = 379, and *A. ovalis* (b), n = 211, from the eastern shore of Virginia. See text for equations.

average of about 78% of his catch with mechanical tongs, and blood clams (*N. ponderosa* and *A. ovalis* combined) 22% for the period of September to November 1994. The percentages of blood clams in catches for those three months were about 18% for September, 21% for October, and 26% for November. The species distribution was similar to that from the field survey samples (i.e., about 72% *Mercenaria* and 28% blood clams). Average daily catches (Fig. 11) for September, October, and November, 1994 were: 4,373, 3,873, and 3,642 clams per day, respectively.

The estimated average catch per unit effort (i.e., clams in one tonging effort, covering 1.12 m^2) during two days of fishing was 9.72 (n = 540) and 3.6 clams (n = 720), respectively, for an overall mean of about 6 clams per tonging effort (n = 1,260). This equates to an average density of 5.4 clams m^{-2} in the harvest area. The average heights of *A. ovalis* and *N. ponderosa* from subsamples of September to November catches were 34.0 mm and 44.7 mm, respectively (Figs. 12a and 12b). The mean height for *N. ponderosa* was less than that for the commercial fishery sample taken in 1992 (70 mm) and the Parting Creek sample (56.0 mm) taken in 1993. The decrease in average size may indicate that overfishing is occurring.

During December 1994 and January 1995, Mr. Bishop worked in an area just north of Wachapreague, VA (Gargatha Creek) and reported catching almost all *A. ovalis*, the highest percentage catch of that species of which we are aware. His observation is noteworthy because of the normally small percentage of *A. ovalis* in catches, and indicates that small, dense beds of *A. ovalis* exist in some isolated locations. The average height for *A. ovalis* from the January sample in Gargatha Creek was 40.2 mm (Fig. 12c). Most of the clams were in the 30–40 mm size range (= 33 to 44 mm in length), or >3+ years old. The absence of 0+ year-class *A. ovalis* in commercial fisheries samples may simply reflect the difficulty in seeing and collecting very small clams in the mud and debris which accompany catches, or it may also indicate low recruitment and/or high mortality rates. Another, more probable explanation is the fact that small *A. ovalis* are epiphytic. During our study, the easiest place to find 0+ year-class *A. ovalis* was enmeshed in bryozoan and hydrozoan colonies attached to commercial mollusc floats near Quinby or Wachapreague Inlets.

CONCLUSIONS

Survey data provide information about the species composition of the blood clam fishery on the Eastern Shore of Virginia. The hard clam, *M. mercenaria*, constitutes the majority of the catch (72%), with *N. ponderosa* accounting for about 17%, and *A. ovalis*

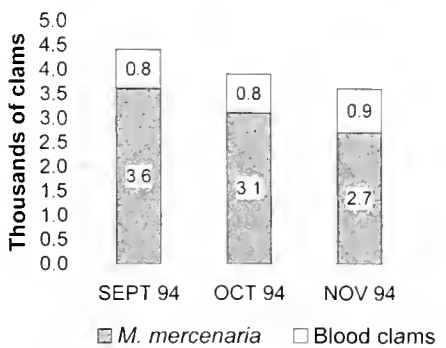

Figure 11. Average daily catch of blood and hard clams (*M. mercenaria*) by a fisherman on the eastern shore of Virginia (Sept.–Nov. 1994).

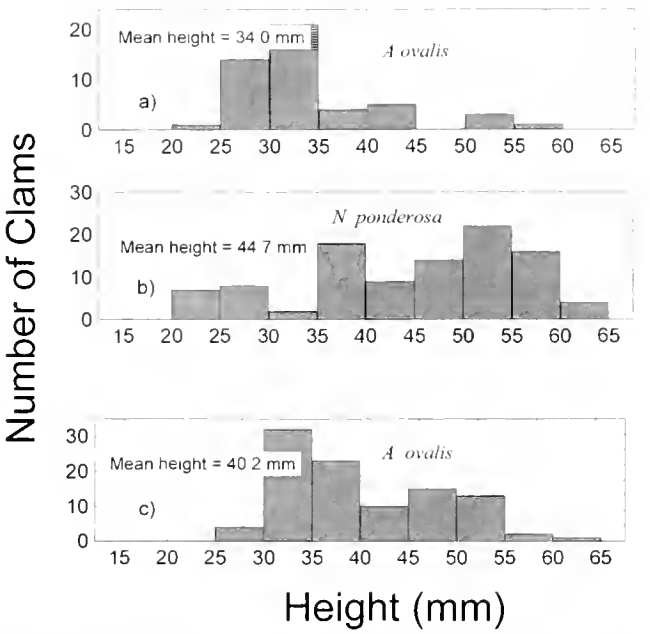

Figure 12. Height frequency distributions for *A. ovalis* (a) and (c) and *N. ponderosa* (b) from Quinby and Wachapreague, Virginia.

11%. However, age-length relationships clearly show that *N. ponderosa*, even though it is more abundant than *A. ovalis*, is a relatively slow-growing species, and may not be suitable for a commercial fishery with high exploitation rates. *Anadara ovalis* grows faster but appears to have a high mortality rate in most areas, as indicated by the relatively few, small ones that were taken in survey samples, the low percentage taken in commercial catches, and field experiments (McGraw et al. 1998).

We estimated abundance for *N. ponderosa* and *A. ovalis* to be about 16 million and 6.4 million clams, respectively, in the general area surveyed. Catch estimates range up to 1.5 million blood clams harvested annually in the survey area. Although this is about 15% of the estimated abundance, we are concerned that if fishing continues unabated, blood clam populations could be decimated within a few years, particularly in light of data showing that *N. ponderosa* grows so slowly and observations that settlement is very sporadic. Survey data as well as samples of commercial landings from the Great Machipongo River and other areas show a decrease in average size of *N. ponderosa*, indicating that *N. ponderosa* is currently being overfished and that there is a need to re-evaluate current policies governing the blood clam fishery on the eastern shore.

Given the distinct possibility of overfishing, we think one of the best conservation measures with regard to blood clams is the cultivation of *A. ovalis*. It has a comparatively fast growth rate, should be relatively easy to spawn under hatchery conditions, and could be grown in conjunction with *M. mercenaria* on existing leases. We have anecdotal evidence that natural set of blood clams is sporadic and undependable, but hatcheries could provide a reliable source of seed. In addition, effective, feasible methods of floating culture (i.e., "Taylor floats") are already in use for hard clams on the eastern shore (Luckenbach and Taylor); similar use for *A. ovalis* would substantially reduce predation and enhance survival rates. If *A. ovalis* can be successfully cultured, this may augment or supplant the harvest of blood clams by other means and, possibly, lessen some of

the fishing pressure from them. Otherwise, the blood clam population on the eastern shore may decline rapidly over the next several years.

ACKNOWLEDGMENTS

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A DIGITAL PRESENTATION OF THE MARYLAND OYSTER HABITAT AND ASSOCIATED BOTTOM TYPES IN THE CHESAPEAKE BAY (1974–1983)

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ABSTRACT Between 1975 and 1983, the Maryland Department of Natural Resources conducted a survey of Maryland's portion of the Chesapeake Bay to reassess the extent and condition of oyster bottom (bars) that were initially surveyed in 1912. A variety of methods were employed to assess bottom condition, including bottom grabs, sounding poles, and dragged microphones. The survey used six categories to describe substrate type. Three of these represented oyster bottom: cultch (exposed oyster shell), cultch with sand, and cultch with mud. The other three categories were non-oyster bottom: sand, mud, and consolidated hard sediment. Bottom characterizations were drawn on 37 transparent *Mylar* sheets. Although the resulting charts were used to generate new legal oyster bar boundaries, the original bottom characterizations were never published. We present here the digitized *Mylars* in a Geographic Information System (GIS) polygon format, which is now available as a digital file. A comparison of results with more recent habitat assessments indicate the survey was a generally valid representation of the true bottom condition. The digitized survey results, however, could be misapplied if used as an exact description of current bottom conditions, because in many areas, cultch categories are actually shell covered by varying depths of sediment. This work provides a demonstration of how GIS-based analysis can be employed to interpret historical surveys of oyster habitats and provide a useful management product to resource managers.

KEY WORDS: oyster, habitat, Chesapeake Bay, GIS, cultch

INTRODUCTION

Fisheries management and ecological restoration of oyster habitat are generally obliged to rely upon old original bottom surveys, often over 100 years old. These surveys covered broad areas but relied upon crude methods. In the Chesapeake Bay, which lies within the boundaries of Maryland and Virginia, broad scale maps of oyster (*Crassostrea virginica*) habitat were constructed in the late 19th and early 20th centuries. In Virginia, oyster habitat was mapped by Baylor (1894). The first official survey of Maryland oyster bars was referred to as the Yates survey. Completed in 1911 (Yates 1911, Smith 1997), it was authorized in 1906 (Kennedy & Breisch 1983) to delineate oyster bottom to be reserved for public oyster harvest. A dragged chain was employed as the principal sampling method for the survey. The survey was in response to declining oyster harvest which went from 686,700 m^3 of live oysters in the shell in 1884 to 201,432 m^3 in 1906, despite increased harvest effort (Graves 1912). In the years following the Yates survey, much bay bottom was added to the public oyster ground by judicial decree. By the 1970s, there was increasing opinion that much of the original Yates-designated public bottom had become unproductive and should no longer be reserved exclusively for harvesting of oysters from public beds. Alternative uses were proposed for the public bottom, which by 1974, produced only 128,184 m^3 (MD-DNR 1989) of oysters annually (8,294 metric tons, NMFS 2000). Fishers interested in harvesting soft clams (*Mya areneria*), in particular, called for a re-examination of the original survey to determine which areas were still productive public oyster beds, which areas could be designated for clamming, and which could be available for private oyster leases. In 1974, the Maryland State Legislature authorized the Maryland Bay Bottom Survey (MBBS). The original mandate for the survey was to delineate the natural oyster bars of the State, the soft clam areas of the State, and what will be termed barren bottoms of the State, which could be leased to private individuals for oyster culture or other aquaculture purposes.

The challenges presented by the need to map oyster habitat and track historical changes are formidable. In Maryland, survey methods have varied greatly since the Yates Survey. The MBBS employed a combination of mechanical grab samples and hydroacoustic techniques to classify bottom types. The output from the MBBS were bottom-type renditions, represented as polygons, drawn on a series of transparent *Mylar* sheets. Each transparency matched the scale of one of the 37 official State of Maryland Natural Oyster Bar (NOB) charts (MD-DNR 1983) that depicted the boundaries of Maryland's public oyster bars. Although they were drawn at the scale of Maryland oyster bar charts, no reference information, such as latitude and longitude or shoreline, was depicted on the sheets. As such, they were extremely difficult to utilize in their original format. Because these renditions were not geographically referenced, and because they were gross simplifications of the information generated in the survey, they fell short of providing a comprehensive tool to manage the resource and track historical change. Instead, the principal use of the MBBS transparencies was to produce new legal oyster bar boundaries. In conjunction with the older Yates survey, the primary criteria for designation of new oyster bar boundaries was the distribution of cultch (exposed oyster shell)-bearing bottom. Because of the physical complexity of the bay bottom, new legal oyster bar boundaries were negotiated gross simplifications of bottom depicted by the MBBS. The first of the new NOB boundaries became effective in 1982. After serving as a basis for the generation of new official NOBs, the 37 *Mylar* sheets depicting the MBBS were archived.

In 1994, the Chesapeake Bay Stock Assessment Committee provided funding to digitize the original MBBS data into a GIS format for future public dissemination. GIS technology allows these data to be integrated with other spatially related datasets, such as bathymetry, for purposes of resource assessment. This paper was prepared in association with the introduction of the MBBS digitized dataset to the public. Because no detailed and integrated documentation of this survey was ever prepared, this review serves as a description and analysis of survey methods and results. In an effort to track historical changes in the oyster re-

source and habitat, and to provide for more effective management and restoration activities, it can be seen from this effort that there is a need to develop quantitative tools for comparing historical and current or future surveys of oyster habitat. From this process, we have identified approaches to applying quantitative Geographic Information System (GIS) analysis and display to plan for new approaches to oyster habitat mapping.

METHODS

Survey Overview

The survey began in 1975 and was expected to require approximately 3 years to complete. The original objectives stated that the entire Maryland area of the Chesapeake Bay would be surveyed, a departure from the original Yates Maryland oyster bar survey (Fig.1), where local "experts" identified potential oyster bottom for survey (Graves 1912). Because of the slow pace of work, the scope of the survey was later changed to assess only bottoms within and adjacent to the public bars identified by the Yates survey.

Navigation was principally by Raydist radio receivers (Teledyne Hastings, Hampton, VA.) Transmitting stations were established especially for the MBBS, and Raydist control sheets were prepared for boat use by overprinting existing natural oyster bar charts with overlapping hyperbolic Raydist lanes. For most sampling cruises, tracks directly followed Raydist lanes, thus transect bearings varied throughout regions of the bay. Sextant triangulation was used when electronic navigation failed.

Bottom sampling methods varied throughout the survey and included combinations of patent tong grabs (Chai et al. 1992), sounding poles, echo sounder tracings, and sounds from a dragged microphone. Patent tongs are a commercial oyster harvest device employing toothed jaws and a steel-framed enclosure capable of retaining oysters of commercial size. Patent tongs collected material from 1 m^2 surface area. Individual sample depth was variable depending upon substrate composition. It is estimated that approximately 15% of the survey was conducted with patent tongs, 10% with sounding poles, and the remaining 75% with microphones. Table 1 provides phases of the survey in which particular sampling methodologies were employed. Maximum MBBS survey depth was about 9 m because of limitations of patent tong sampling; boat draft limited minimum surveying depth to about 2 m.

All field surveying, with the exception of the Potomac River,

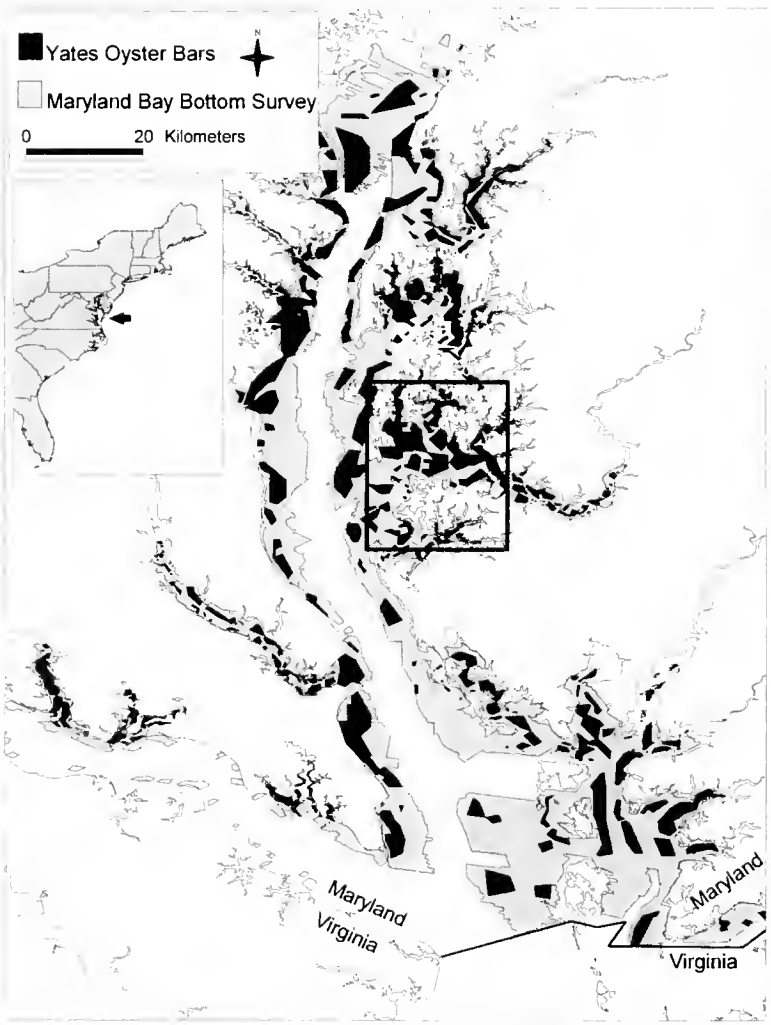

Figure 1. Comparison of the area covered by the original Yates oyster bars (black) to the region of the Chesapeake Bay covered by the Maryland Bay Bottom Survey (gray). Yates bars were surveyed in 1911 to delineate grounds for public oyster harvest. The boxed region is the lower Choptank River represented in Figure 2.

TABLE 1.

A chronology of sampling methods used by Maryland Bay Bottom Survey[a].

Method	Transect lane spacing	Station spacing along lanes	Data collected	Estimated km[b] sampled/day
Phase I–early 1975 to late 1976 Patent tongs (1 m^2), bottom profiling recording fathometer, and sounding poles	137–183 m	137–183 m	Bottom type and presence of benthic organisms	1.42
Phase II–late 1976 to early 1977 Recording fathometer only[b]	137–183 m	137–183 m	Three bottom types: hard, soft, unknown	2.02
Phase III–summer 1978 to late 1979 Dragged microphone[c] with shipboard transcription, recording fathometer, and sounding poles	183 m	183 m	Seven bottom types: sand, mud, shell/sand, shell/mud, shell, clay, and gravel	1.62
Phase IV–February 1980 to May 1980 Dragged microphone recorded shipboard with transcription on shore	183 m	Continuous	Six bottom types: sand, mud, shell/sand, shell/mud, shell, hard bottom	11.33
Phase V–June 1980 to November 1983 Dragged microphone analog output recorded on fathometer paper trace	183 m	Continuous	Six bottom types: sand, mud, shell/sand, shell/mud, shell, hard bottom	11.33

[a] Adapted from an anonymous and undated table titled "History of B.B.S. in Maryland" found in Maryland Department of Natural Resources files.
[b] Survey speed was to be increased by eliminating surveying in "nonproductive areas" and to use echo-sounding as an "exploratory/sample investigation." It was hoped that rapid preliminary reconnaissance with the bottom sounder and sounding pole could identify cultchless substrate that would not require exhaustive patent tong sampling. Reconnaissance data were apparently used as a basis to focus where more exhaustive sampling would be employed at a later date.
[c] This instrument was a waterproofed microphone dragged along the bottom that transmitted sound to an amplifier and speaker on the survey boat. Although specifications for the exact design are not available, the configuration described by Haven et al. (1979) would have operated in a similar fashion. Event marks were placed in the location record where there were marked changes in the sound produced.

was completed by October 1982. Survey of the Potomac began in July 1983 and was completed in November of 1983. This portion of the survey was conducted independently of the MBBS, because the Potomac River fishery is jointly managed by the States of Virginia and Maryland. Oyster bars on both shores of the river were surveyed, but oyster bars in Virginia tributaries were not charted. Preparation of bottom charts continued until the end of 1983.

The total area surveyed by the MBBS was 2591.9 km^2 and covered 42% of the Maryland portion of the Chesapeake Bay. A total of 7,543 polygon objects were used to depict bottom type (Fig. 2). Of the total area surveyed, 1,858 km^2 was outside of the original charted Yates bars. In the total survey, 477.7 km^2 of bottom was cultch, 201.9 km^2 sand with cultch, and 205.2 km^2 mud with cultch (Table 2). The GIS generated results included 0.575 km^2 of designated leased bottom, an attribute characterization for which habitat classification is not available.

TABLE 2.

Summary of bottom-type polygons digitized from original Maryland Bay Bottom Survey Charts.

Bottom type	No. polygons	Area (km^2)
Sand and shell	1,999	201.9
Shell	1,958	477.7
Sand	1,177	838.5
Mud and shell	1,155	205.2
Mud	845	733.4
Hard bottom	409	135.2
Total	7543	2591.9

Digitization

We digitized MBBS *Mylar* charts individually with MapInfo® software and then merged them into one datafile. The MapInfo projection and datum used was Longitude/Latitude (NAD27). Twelve to 15 control points were used to register the map to Earth coordinates. Coordinates of control points were georeferenced by overlaying the MBBS *Mylar* over the corresponding Maryland Natural Oyster Bar chart that had a graticule of latitude and longitude. Control point error values were all less than 0.05 cm root mean square in true chart dimension. Bottom type on MBBS mylars was represented as inked shapes, shaded to depict six discrete bottom categories. These categories were: cultch, sand with cultch, mud with cultch, sand, mud, and consolidated "hard" sediment (believed to be clay). Hand-drawn, smooth-sided delineations on the *Mylar* sheets were transformed to straight-sided polygons in the digitization process. We attempted to duplicate the original curved-sided objects as closely as possible by utilizing enough segment nodes to make the straight-sided polygons appear curved at normal magnifications of the GIS image (Fig. 2). Final data cleaning was performed to identify sliver polygons where two objects overlapped. This and other cleaning procedures were performed in ArcInfo® utilizing the "build" and "clean" functions.

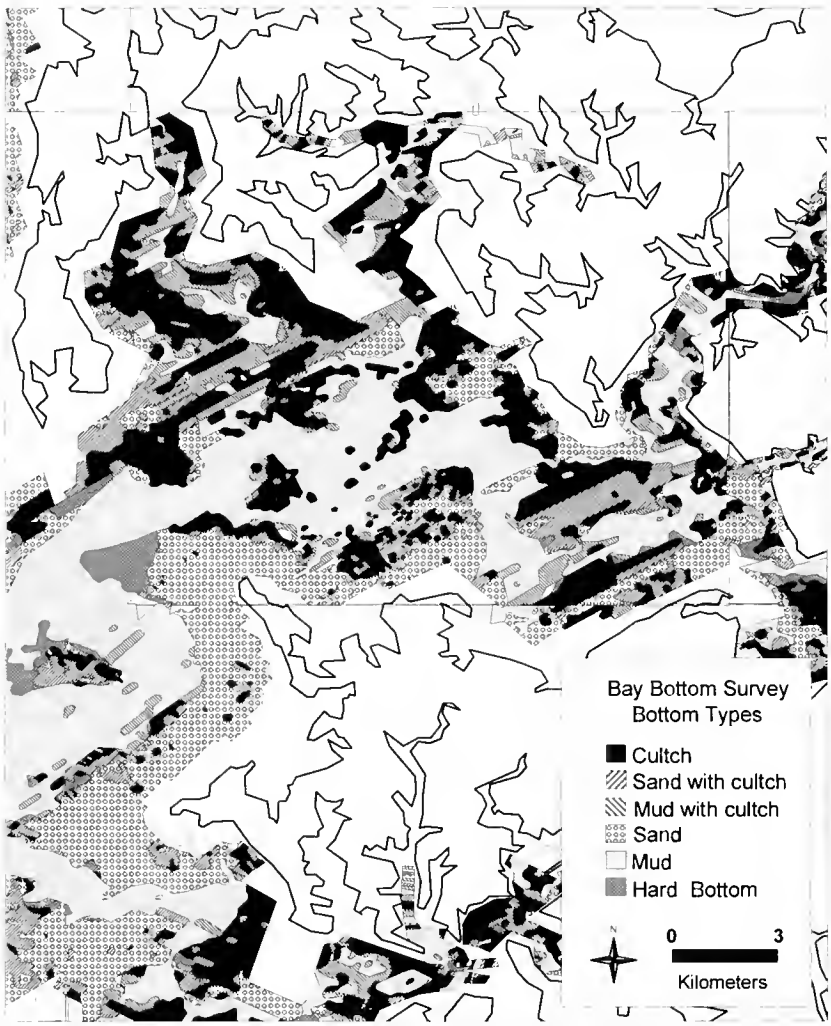

Figure 2. An example of the digitized rendition of the Maryland Bay Bottom Survey in the Choptank River region. The shoreline and the borders of the original *Mylar* charts are layered upon the survey bottom themes, but are not included in the digital file. Original *Mylar* transparencies were 70 × 111 cm, drawn at a scale of 1:20,000 and projected in U. S. State Plane NAD27. The general North–East / South–West orientation of bottom themes depicted here is the result of radio beacon navigation.

One of the original 37 *Mylars*, (southern East Bay) was missing from the set before digitization. Summary statistics do not include this lost *Mylar*, and the charted area it occupies in the digital dataset is empty.

Validation of Habitat Characterization

Comparative Analysis

To determine the relationship between categorical bottom types of the MBBS depictions and raw patent tong data from which they were generated, we obtained original field data from 1975 for two oyster bars, Cook's Point and Sandy Hill (Fig. 3), in the Choptank River. These oyster bar locations were chosen from a limited set of raw data still available from the original survey. We georeferenced and then superimposed this raw categorical bottom type data over the MBBS habitat depictions. Categorical bottom types identified on the patent tong datasheets included shell, sand and shell, mud and shell, sand, mud, mud and sand, stone, and clay. These are consistent with the MBBS bottom objects for these same locations.

In addition to categorical bottom type data, survey sheets gave the percentage of the patent tong sample by volume, which was cultch. To validate further how final survey bottom classification were made, we evaluated the association between cultch quantity in patent tong samples and MBBS habitat categories present at the two oyster bars. Data for individual samples from both bars were pooled, and a square root transformation on cultch percentages was performed after an evaluation of residuals. Data were subjected to a one-way analysis of variance (ANOVA), with bottom type as the experimental variable and percentage cultch as the response variable ($\alpha = 0.05$). A Tukey's mean comparison test (HSD) was then performed at $\alpha = 0.05$.

We also compared 1975 MBBS patent tong samples from Sandy Hill and Cook's Point to unpublished (M. Homer pers. comm.) 1993 patent tong samples obtained from the same bars by the Maryland DNR Oyster Stock Assessment Program (OSAP). Unlike MBBS datasets, OSAP sampling was uniform and complete within the entire Yates bar boundary. OSAP data were recorded as the number of liters of surface shell in each sample; whereas MBBS data were recorded as the percentage of shell in each sample. Because the two sampling methods were not in comparable units, nor convertible, cultch densities for both percentage

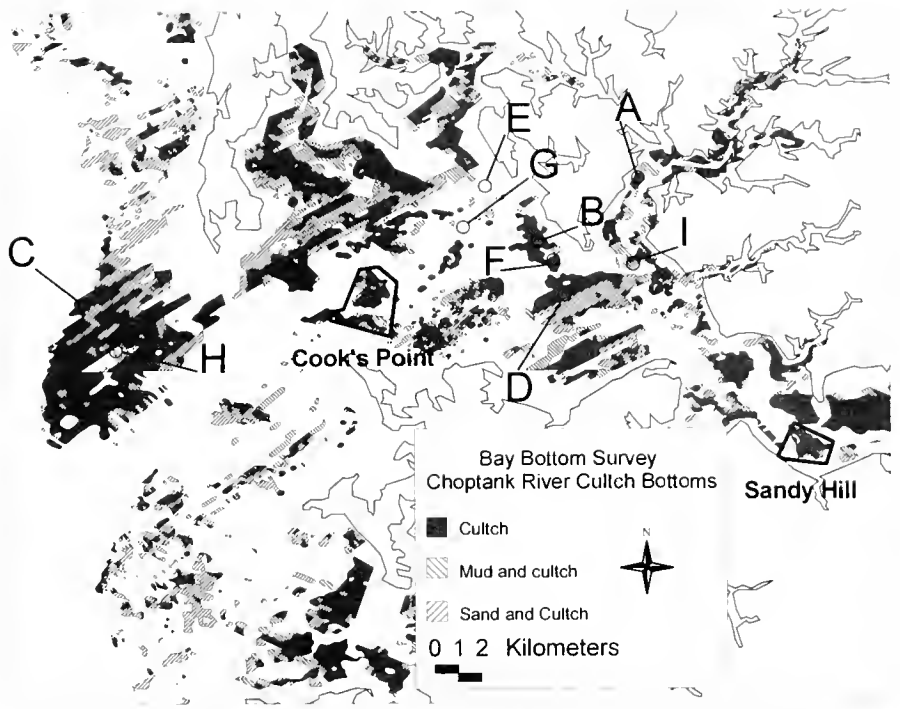

Figure 3. Selected diver observation and sediment analysis sites located on Maryland Bay Bottom Survey cultch designated bottoms in the Choptank River. Results of bottom analyses from sites A–H are presented in Table 3. Yates bar boundaries outline Cook's Point and Sandy Hill oyster bars.

of sample and liters per sample were each standardized to frequency distributions with a mean of 0 and an SD of 1.

Patent tong data from OSAP sampling at Cook's Point and Sandy Hill were also used to validate the shape of MBBS cultch objects. We created a continuous surface from OSAP data by importing point datafiles into an ArcInfo grid cell model. Cultch density patterns in the grid were then compared to the shape of cultch objects in the MBBS.

We used SCUBA dives in 1998 to perform qualitative assessment of the abundance of surface shell, and subsurface composition by probing with a diver knife, at nine locations designated as cultch in the MBBS (Fig. 3). At each site, SCUBA divers also collected one to three two-liter sediment samples to a depth of 15 cm. Samples were weighed and then sieved through 33-mm, 10-mm, and 5-mm mesh to determine percentage of shell by weight in each sample and the composition of nonshell material. Material passing through the 5-mm mesh was then subjected to traditional grain size analysis down to 16 μm (ASTM 1990).

An underwater benthic sled with color and B/W cameras was towed across the nine locations from where we collected the sediment samples. The direction of the video observation was variable, largely based upon wind and current condition. But, the exact path traversed was logged directly on the video tape by differential GPS signal.

Sub-Bottom Profiling

We used acoustic sub-bottom profiling systems to determine the relationship between MBBS cultch objects and Yates bar boundaries to oyster bar geomorphology. Transects were taken in January 1998 with an X-star® sub-bottom profiling system (Edgetech Inc., Milford, MA, USA) on and adjacent to Sandy Hill and Cook's Point oyster bars. Sub-bottom profiling output was a GPS logged gray-tone trace that identified bottom depth and the presence and depth of sub-bottom density discontinuities. Because output was GPS logged, we were able to associate profile features with MBBS objects.

RESULTS

Comparative Analysis

At Cook's Point and Sandy Hill oyster bars, only four of the six MBBS bottom classifications were recorded (Figs. 4a, b). Survey bottom polygon characterizations generally corresponded to raw patent tong bottom-type categorical data at Cook's Point and Sandy Hill bars. Seventy-eight percent of patent tong sample points were bounded by corresponding bottom type polygons at Cook's Point and 71% of points corresponding to like-bounding polygons at Sandy Hill. One apparent discrepancy in MBBS bottom characterization at Cook's Point was that patent tong datapoints characterized as sand with cultch or mud with cultch were depicted within bottom- type objects defined as cultch. Similar inconsistencies were found at the Sandy Hill site.

Mean percentage cultch varied significantly among the five categorical bottom types at the two sites. Tukey's mean comparison test indicated that mean percentage shell was significantly lower in the mud and sand bottom designations than in the cultch and sand, cultch and mud, and cultch categories (Fig. 5). Cultch density differences among the cultch, sand with cultch, and mud with cultch categories were not significant. These results indicate that the MBBS used qualitative assessment methods, and not strictly quantitative, to categorize cultch bottom types.

Analysis of OSAP data at Cook's Point and Sandy Hill provided little insight on the accuracy of MBBS bottom classifications and shapes of chart objects. Standardized cultch densities from the two surveys had differently shaped frequency distributions (Fig. 6). Cultch distributions at Cook's Point and Sandy Hill oyster bars

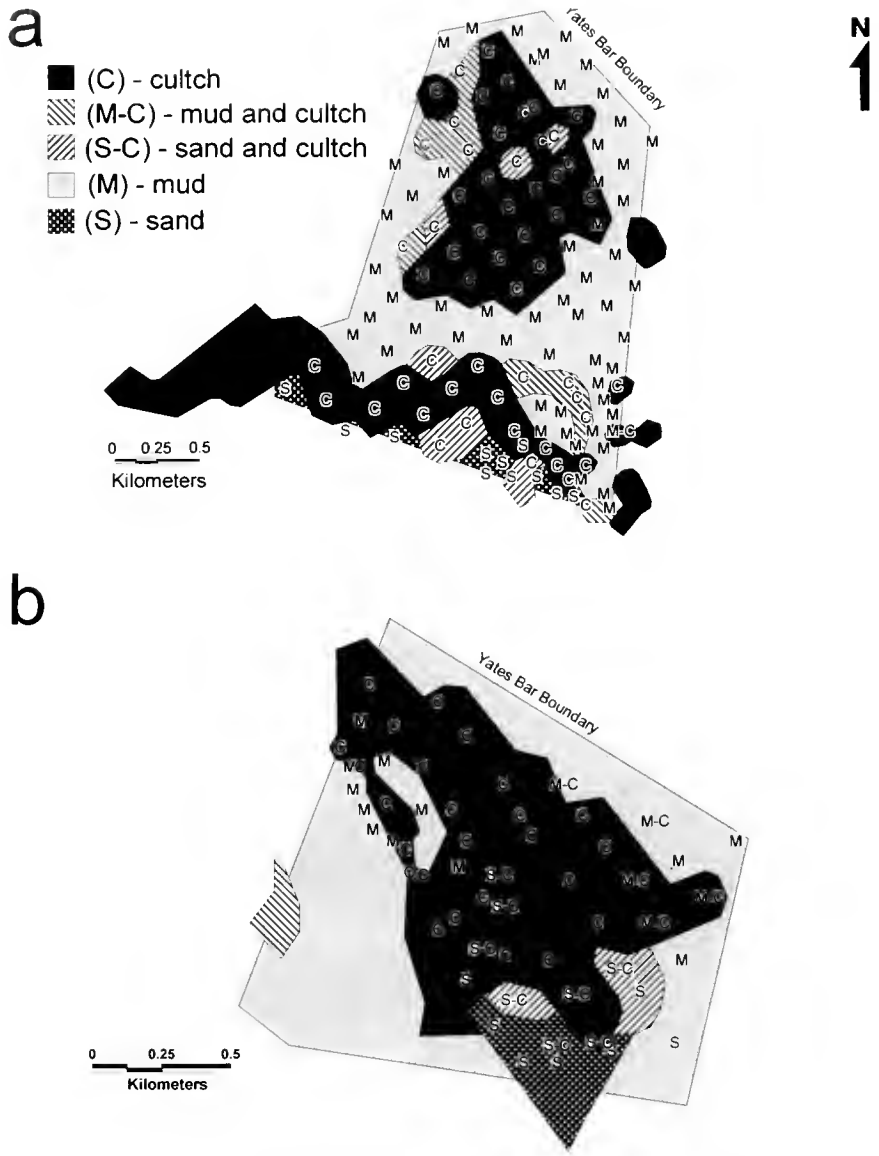

Figure 4. Relationships between patent tong point data and final bottom-type polygons of the Maryland Bay Bottom Survey. Point data are layered over bottom themes within Yates Bar boundaries at (a) Cook's Point and (b) Sandy Hill oyster bars. Characters represent substrate type: C = cultch, SC = sand and cultch, MC = mud and cultch, S = sand, and M = mud. Irregular spacing of datapoints within the Sandy Hill Yates bar boundary are attributable to lost data sheets.

from 1975 (MBBS) indicated a majority of low-density samples, with some high-density samples being present. Later 1993 (OSAP) data was highly skewed to low-density samples, with no high-density samples collected. Differences in shell density distributions indicate that either MBBS patent tong sampling was imprecise, relative to OSAP sampling, or that there has been a temporal decline in cultch density at the two oyster bars over the intervening two decades.

We did not observe any relationships between shell density patterns from OSAP grid cell models and the shape of MBBS cultch polygons. Multiple displays at various shell density levels used to depict cultch did not match MBBS cultch object shapes at either of the two oyster bars.

Results of diver observations, video footage, and sediment analysis at selected sites in the Choptank River indicate that much of the bottom described as cultch in the MBBS currently has little surface shell (Table 3). Divers, however, noted dense shell below surface sediments at all but one location. These results indicate that much of the bottom depicted as cultch in the MBBS is currently degraded by sedimentation or that some of the methods used in the MBBS were unable to discern sedimented shell from clean shell.

Sub-Bottom Profiling

We found that the physical perimeters of oyster bars generally occur at points where hard sub-bottom terraces emerge from surface layers of soft sediment or at main channel margins. These features are identified on sub-bottom profiles by acoustic density discontinuities or rapid bathymetric change. Borders of MBBS cultch objects and Yates bar boundaries at Cook's Point and at Sandy Hill generally coincide with these features (Fig. 7). In most cases MBBS cultch boundaries were more accurate in depicting

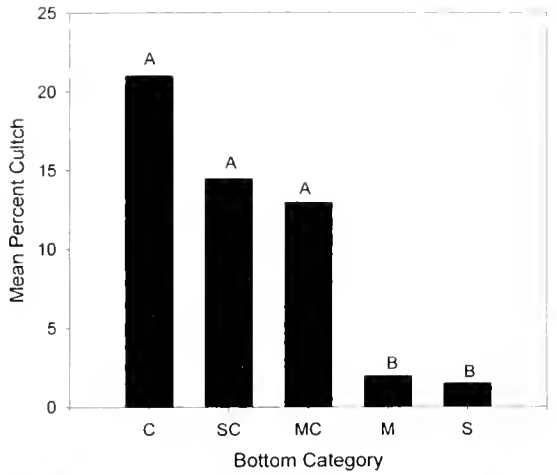

Figure 5. Mean percentage cultch from patent tong samples relative to corresponding bottom classifications. Patent tong data were pooled from collections made at Cook's Point and at Sandy Hill oyster bars. We used analysis of variance (ANOVA) and Tukey's multiple comparison test ($\alpha = 0.05$) on square root transformed data to assess the validity of categorical bottom designations. Mean percentage cultch at bottom types with the same letter were not significantly different. Bottom categories are: C = cultch, SC = sand and cultch, MC = mud and cultch, M = mud, S = sand.

true oyster bar margins than the original Yates bar charts. The generality of Yates bar boundaries results from the purpose of the survey, which was charting protected public oyster bottom rather than identifying bottom types.

DISCUSSION

The 1975 to 1983 Maryland Bay Bottom Survey was the first baywide assessment of oyster habitat since the early 1900s. The area of bottom covered was 342% larger than the portion of the bay designated as public oyster bar by the Yates survey of 1911. Unlike the Yates survey, the MBBS is a categorical description of bottom that is relevant to oyster ecology in addition to regulation of harvest. Our digital rendition of the MBBS survey increases the facility of analysis and display at local and regional scales. The digital format will also allow resource managers and interested parties to identify historic regions of oyster habitat and areas for rehabilitation. This dataset is available for distribution from the Cooperative Oxford Laboratory (Mapping and Analysis Project, Cooperative Oxford Laboratory, 904 S. Morris St., Oxford, MD 21654, (410) 226-0078).

An understanding of the strengths and weaknesses of the MBBS should precede any serious application of these data. Figure 7 is an example of how the MBBS may be a more accurate depiction of the true morphological character of oyster bars than the previous Yates survey. In many cases, the MBBS polygon boundaries corresponded to oyster bar edges identified by sub-bottom acoustic profiles; whereas, Yates bar boundaries provided a more generalized representation. Comparison between bottom category and percentage cultch indicated that bottom classifications, at least where patent tong sampling was employed, were systematically determined from cultch volume and that there was a relationship between MBBS bottom category and cultch density.

Little documented information exists about the methodology and consistency of the methods used to translate field data to the final six bottom classifications. The limited information available from raw patent tong data at Cook's Point and Sandy Hill oyster bars (Figs. 4a,b) showed a relationship of sample data to the derived objects, but there were some discrepancies. These inconsistencies suggested that additional information was incorporated into developing bottom classification. Patent tong field sheets from Cook's Point and Sandy Hill show that data were recorded for bottom type and percentage cultch for upper, middle, and lower strata within each patent tong sample. Although bottom type and percentage cultch were, in many cases, different between strata from the same sample, there was no consistent pattern indicating whether sub-surface information was used to generate final bottom categories.

The graphic representation of the MBBS was affected by data collection and translation procedures. A linear pattern of northeast–southwest orientation can be seen throughout the charts (Fig. 2). This is an artifact of transect orientation along radio beacon lines, and the width of these patterns provides the scale of transect spacing. Because each of the 37 *Mylar* sheets were drawn

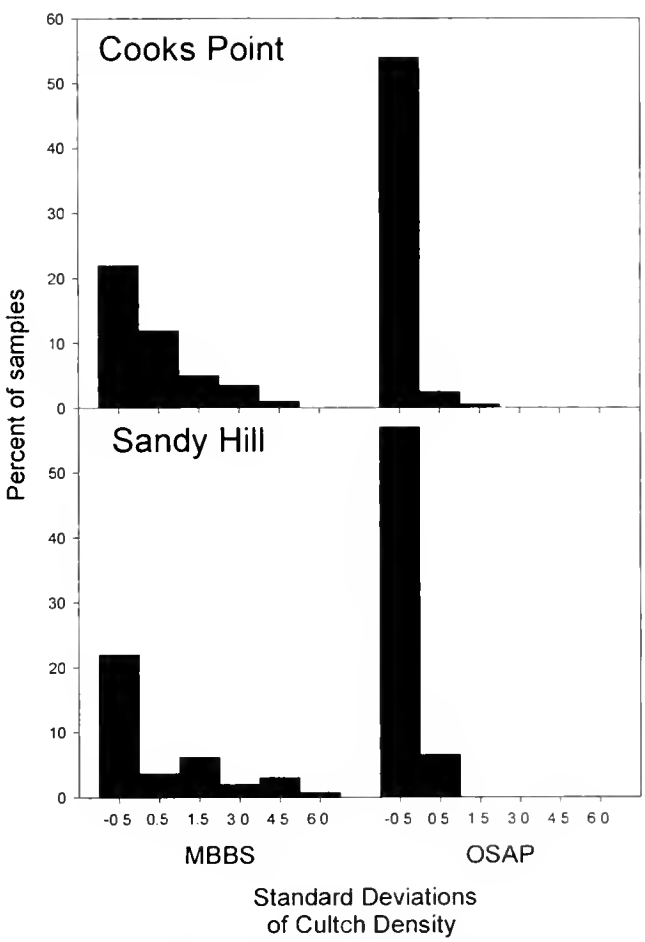

Figure 6. A comparison of cultch densities from 1975 patent tong samples recorded on Maryland Bay Bottom Survey (MBBS) field sheets to patent tong samples collected in 1993 at Cook's Point and Sandy Hill oyster bars. Recent patent tong samples were collected for the MD DNR Oyster Stock Assessment Program (OSAP) and were recorded as liters of shell per sample; whereas, MBBS data were recorded as percentage shell per sample. To compare absolute and relative units, both datasets were fit to standard frequency distributions with a mean of 0 and an SD of 1.

independently of each other, objects at chart edges did not merge precisely, and we were forced to adjust most junctures when we integrated individual charts into the final GIS data file (Fig. 2).

For some information requirements, the MBBS may not be a realistic depiction of the conditions of the Chesapeake Bay bottom in the 1970s and 1980s. Most bottom areas observed in the mesohaline portion of the bay are transitional, grading between the six generic categories of the MBBS. Intermediate bottom types often predominate over discrete bottom types, and many areas of the bottom, particularly where cultch is present, exhibit extreme heterogeneity. The categorical polygon data model used does not represent transitional areas, because it depicts an abrupt change between bottom types. Furthermore, depictions of bottom, are for the most part, characterizations assembled from sampling points along a transect line, thus fine scale heterogeneity is not adequately represented. Our diver and video observations on the bottom in the vicinity of Cook's Point and Sandy Hill oyster bars show extreme patchiness of cultch and sediment at centimeter and larger scales. An additional complication is that we do not know exactly what the "hard bottom" MBBS bottom habitat classifications means. The term "deep water" has been associated with it in some reference notes, although the survey did not extend into waters deeper than 9 m. Our visual observations in hard bottom areas often show a clay-like substrate.

Current Relevance

Most of the MBBS survey was conducted mainly with a dragged underwater microphone, and identification of cultch was generated by contact and abrasion on hard objects. Little information, however, is available to determine the accuracy of MBBS microphone data. Verification sampling via patent tong and diver observation to validate microphone acoustics was initiated toward the end of the survey, but results cannot be located. A principal source of error associated with the microphone method is that oyster shell is not the only hard substrate on the bottom. We have observed pebbles, cobbles, and occasionally boulders from video and diver observation in the Choptank River region. Some of these stony bottoms often include scattered or dense shell and were characterized as cultch in the survey. We know that the microphone distinguished smooth from rough bottom and could identify transition zones between the two. However, the presence and density of cultch may be confounded by other hard objects. The use of an underwater microphone for surveying was not novel. From 1978 to 1981 the public oyster grounds of the James River in Virginia were similarly surveyed (Haven et al. 1979, Haven & Whitcomb 1983), with data being reduced to six bottom characterizations identical to those later employed by the MBBS.

Acoustics were also employed in the mapping of Galveston Bay, Texas beginning in 1991 (Powell et al. 1995). In this case, dual frequency transceivers (27 and 300 kHz) were utilized to compare results to data collected in the 1970s by a manual polling technique. Polling was additionally employed as ground truthing during the recent survey. Principal oyster habitat distinctions employed were between hard consolidated reef and unconsolidated shell. GIS data generation were employed principally to generate polygon areas of these categories. Unlike the Maryland Bay Bottom Survey, wholesale habitat type mapping was not a component of the published results.

Our analysis of sediment samples, qualitative diver observations, and video footage, at areas characterized as cultch by the MBBS showed small amounts of surface cultch and an almost universal presence of shell beneath surficial sediments. Trace quantities of exposed cultch or no exposed cultch was the dominant surface character. This situation is true for almost the complete extent of the large MBBS cultch object depicted at the top of Cook's Point oyster bar (Fig. 7). Video transects over tens of meters did not yield any surface cultch. These observations seem to agree with results of the OSAP patent tong-sampling program from the early 1990s. From the OSAP data, we calculated a mean shell volume of $1.6 \times L \times m^{-3}$ from samples collected within all MBBS cultch, sand with cultch, and mud with cultch objects within Yates bar boundaries for the entire Choptank River region. When completely filled, patent tongs sample 52.0 lm^3 of substrate in each grab. The standardized rank comparisons in Figure 6 also indicate that much of the bottom depicted as cultch in the MBBS

TABLE 3.

Recent substrate examinations at sites identified as cultch bottom by the Maryland Bay Bottom Survey 1975–1983.

Site	Bottom category	Mean shell abundance (% by weight)	Qualitative visual estimates		
			Shell abundance (%)	Nonshell[a] substrate type	Subsurface composition
A	Cultch	7	0	4	Dense shell
B	Cultch	5[b]	50[b]	2,3,5,6,7	Shell/rock
C	Cultch	6	1	1,2,3,5,6,7	Shell/rock
D	Cultch	8	15	4,6	Dense shell
E	Cultch	9	1	3,4,7	Dense shell
F	Cultch	0	1	7	Light shell
G	Cultch	16	0	4	Dense shell
H	Sand/cultch	0	1	3,7	Dense shell
I	Mud/cultch	0	0	4	Mud

[a] Substrate codes: 1 = boulder, 2 = cobble, 3 = grit, 4 = mud, 5 = pebble, 6 = shell bits, 7 = sand.

[b] Difference in shell abundance due to extreme bottom heterogeneity at site.

Sites were selected to be representative of the widest variety of bottom types observed in the Choptank River estuary of the Chesapeake Bay. One to three replicate sediment samples were collected by divers at each site in summer of 1998. Shell abundance was measured as percentage by weight of shell >30 mm from 2 L samples taken to a depth of 15 cm. Visual estimates were recorded from qualitative diver observations of surface shell abundance, nonshell substrate composition, and subsurface composition determined from probing with a diver's knife. Site locations are depicted in Figure 3.

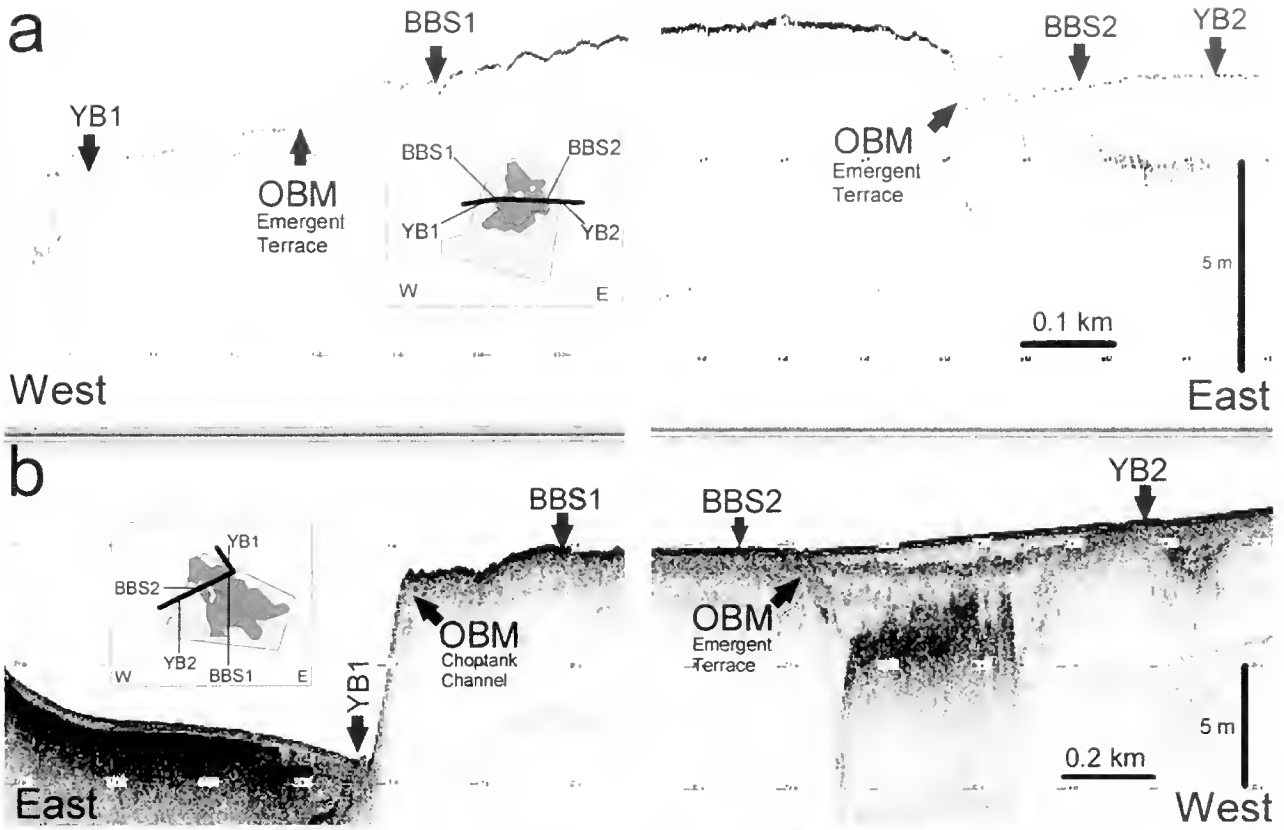

Figure 7. Acoustic sub-bottom profiles taken at Cook's Point (a) and Sandy Hill (b) oyster bars. Arrows indicate where boundaries of Maryland Bay Bottom Survey cultch objects (BBS1 and BBS2) and Yates bar perimeter boundaries (YB1 and YB2) intersect sub-bottom profiling transects. Inserts show where transects cross MBBS cultch objects and Yates bar boundaries. Actual oyster bar margins (OBM) occur where hard sub-bottom terraces emerge from soft bottoms or at the edges of existing channels. The western margin of Sandy Hill occurs at the edge of a sediment-filled paleochannel of a tributary of the Choptank River. The dark feature in the channel is the signature of gas-charged sediments.

is currently degraded by an overburden of sediment. We cannot determine whether this is a recent phenomenon or existed in the mid-1970s to 1980s when the MBBS survey was performed, and methods used by the MBBS may have been biased against detecting sediment on shell. Furthermore, the underwater microphone may have been unable to distinguish clean surface cultch from cultch covered by a layer of sediment. Also, sampling the bottom with patent tongs allows any overlying sediment to be washed from shell as the sample is brought up through the water column.

Caution must be used in how the results of the MBBS are applied. Comparison between the Yates survey and MBBS charts have been used to estimate temporal decline of oyster habitat (Rothschild et al. 1994). Although this study implicates that overharvest has an impact on the oyster habitat, comparative methods between the Yates boundaries and MBBS polygons did not include the MBBS bottom category mud with shell as oyster bottom. Aerial summations were only made with the shell and sand with shell categories, thus potentially greatly inflating habitat loss estimates (Table 3).

In addition, we have shown that Yates bar boundaries may provide coarser descriptions of the distribution of shell resources than the MBBS. This is because the Yates survey was intended to map legal boundaries of public oyster bottom and not necessarily shell distribution. MBBS cultch objects reveal the distribution of shell on the bottom, but say nothing about the quality of oyster habitat. Because oyster veligers require a clean surface for settlement, sediment-free shell is one of the most important components of quality oyster habitat (MacKenzie 1983). Patent tong and underwater microphone methods used to assess oyster habitat may also be unable to identify clean shell from that carrying a sediment load. To assess temporal changes in oyster habitat accurately, new methods must be developed to differentiate between clean shell and shell covered by an overburden of silt (Smith and Greenhawk 1998).

The digitization of survey results and GIS analysis of data has shown the value of GIS as a tool for the examination and analysis of benthic data. This complete dataset of over forty chart size *Mylars* can now be readily examined at ease on a computer screen in association with such related datasets as shoreline, bathymetry, and recent or planned sonar surveys.

Because of the recent integration of acoustic habitat assessment technology with extremely accurate satellite positioning information and GIS data capture and display, new possibilities are available to chart cost effectively and computer generate extensive bottom surveys. The cumbersome nature of data reduction performed in the MBBS generation can now be eliminated.

Benthic habitat charting via remote acoustic methods now provides three principal technological approaches for the assessment and representation of the bottom. Traditional single beam sonar can be used to assess general surface characteristics or sub-surface features. Habitat classification with such systems is still largely subjective. Side scan sonar technology can provide for strip-like textural images of the bottom that can now, by means of computer mosaicking, be seen as large, two-dimensional images. Although

often highly informative as to bottom character at a fine resolution, these side scan systems are still imaging reflectivity of sound only and often require extensive ground truthing. Finally, new technology referred to as Acoustic Seabed Classification Systems (ASCS) has been developed that statistically classifies acoustic echo returns by waveform characteristics into definable habitat types. With ASCS, rapid bottom classification can be made for each sonar ping transmitted. Because reflected acoustic waveforms carry a multitude of substrate information, it may not originally be known just what parameters of the substrate are being used in classification. As with other acoustic techniques, these systems require extensive ground truthing.

Despite technological advances, almost twenty years after its completion, the MBBS is still the best spatial depiction of over-all oyster habitat in the Maryland portion of the Chesapeake Bay. The scale and effort of the undertaking was so great that it will probably remain for some time as our best baseline information source on the distribution of shell resources. Despite weaknesses in documentation of methodology, and sediment overburden in areas described as cultch, we feel comfortable that the survey is a good guide to the location of areas most suitable for oyster bar restoration and a general guide to sediment composition in Maryland's portion of the Chesapeake Bay bottom.

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DIFFERENTIAL DIAGNOSIS OF MIXED *HAPLOSPORIDIUM COSTALE* AND *HAPLOSPORIDIUM NELSONI* INFECTIONS IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*, USING DNA PROBES

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ABSTRACT *Haplosporidium costale* and *Haplosporidium nelsoni* are morphologically similar pathogens of the eastern oyster *Crassostrea virginica*. In the absence of the spore stage, infections of the two species are extremely difficult, if not impossible, to distinguish using traditional light microscopy of stained tissue sections. Species-specific molecular diagnostics were developed for *H. costale* from the small subunit ribosomal DNA (SSU rDNA) sequence. The polymerase chain reaction (PCR) primers amplified a 557 base pair (bp) region of the *H. costale* SSU rDNA, but did not amplify DNA from oyster (*C. virginica*) or from six other haplosporidans (*H. nelsoni*, *H. louisiana*, *H. lusitanicum*, *Minchinia teredinis*, *M. chitonis*, or *M. tapetis*). The DNA probe was used with *in situ* hybridizations of oyster tissue sections to visualize *H. costale* plasmodia and prespore stages; it did not hybridize with oyster (*C. virginica*) or other haplosporidans (*H. nelsoni*, *H. louisiana*, or *Minchinia teredinis*). DNA-based diagnostics for *H. costale*, in conjunction with molecular tools previously developed for *H. nelsoni*, have overcome limitations of histological examination. From *in situ* hybridizations using both probes, some Virginia oysters previously diagnosed with *H. costale* were found to have mixed infections consisting of approximately 80 to 90% *H. costale* plasmodia and 10 to 20% *H. nelsoni* plasmodia. Plasmodia of *H. costale* were not found in epithelial tissue, only in connective tissue. In addition, use of the DNA probe confirmed the presence of *H. costale* plasmodia in Virginia oysters collected in the fall, an unprecedented seasonality for an advanced *H. costale* infection.

KEY WORDS: *in situ* hybridization, small subunit ribosomal DNA, *Haplosporidium nelsoni*, *Haplosporidium costale*, eastern oyster, *Crassostrea virginica*, parasites

INTRODUCTION

Haplosporidium nelsoni Haskin, Stauber, and Mackin (MSX disease) and *Haplosporidium costale* Wood and Andrews (SSO disease) are morphologically similar pathogens of the eastern oyster, *Crassostrea virginica* Gmelin, that occur along the East Coast of the United States. *Haplosporidium costale* is generally thought to be restricted to high salinity bays (>25 ppt) along the open coast from Virginia to Maine; it is rare in the Delaware Bay and in the Chesapeake Bay (Andrews & Castagna 1978; Andrews 1988). *Haplosporidium nelsoni* occurs from Florida to Maine in both estuarine and oceanic habitats where the salinity is greater than about 10 ppt (Haskin & Andrews 1988). Thus, the distribution of the two pathogens overlaps in high salinity areas from Virginia to Maine.

If spores are present the parasites are easy to distinguish because *H. nelsoni* sporulates only in the epithelium of the digestive diverticula, whereas *H. costale* sporulates throughout the connective tissue of most organs (Couch 1967, Andrews & Castagna 1978). Moreover, spores of *H. nelsoni* are about twice the size of *H. costale* spores (Couch 1967). However, in the absence of spores, differentiation of the two parasites is very difficult, if not impossible. According to Couch (1967) plasmodia stages of both *H. nelsoni* and *H. costale* occur in epithelial and connective tissues in both mixed and single infections, so location of plasmodia is not helpful. *Haplosporidium costale* has a very restricted seasonality, with plasmodia present from March through June and spore stages present during May and June (Andrews et al. 1962, Andrews & Castagna 1978). However, plasmodia stages of *H. nelsoni* may also be common during the spring (Andrews 1982). Morphology of plasmodia has apparently been used to distinguish the species, with some difficulty. Couch and Rosenfield (1968) conducted a comparative study of *H. costale* and *H. nelsoni* in Chincoteague Bay, Virginia. They state that diagnoses of the two parasites in living oysters was based on recognition of the plasmodium, but they do not give any criteria used to distinguish the plasmodia of the two species. Mixed infections of *H. nelsoni* and *H. costale* were observed during the same study (Couch 1967), but they were based on the presence of spores of both species. However, criteria for distinguishing plasmodia of *H. nelsoni* and *H. costale* were provided (Couch 1967). They included: nuclear membranes of *H. costale* usually not as sharply defined or distinct as those of *H. nelsoni* and nucleoli (endosomes) of *H. costale* nuclei proportionately larger, less distinct, more diffuse, and more central than nucleoli of *H. nelsoni*. Andrews and Castagna (1978) stated that all stages of *H. costale* average smaller than those of *H. nelsoni*, but they went on to say that no definitive characters have been found with Harris hematoxylin and eosin (HHE) stain to distinguish *H. costale* and *H. nelsoni* plasmodia.

The specificity of molecular diagnostic tools, especially DNA probes used in *in situ* hybridizations, make them ideal for distinguishing morphologically similar species. Such tools are invaluable in elucidating certain ecological aspects of parasites that are difficult using traditional techniques (Burreson et al. 2000). Molecular diagnostic tools have been developed for *H. nelsoni* (Stokes & Burreson 1995, Stokes et al. 1995a). Specific polymerase chain reaction (PCR) primers have been developed for *H. costale* (Ko et al. 1995), but a DNA probe for that species has not been developed. Here we develop molecular diagnostic tools for *H. costale* and use the DNA probe in conjunction with an *H. nelsoni* DNA probe to identify mixed plasmodial infections of the two species. In addition, the molecular tools provided unexpected new information on the seasonality of *H. costale* in Virginia.

MATERIALS AND METHODS

DNA Sequences and Oligonucleotide Synthesis

The SSU rDNA sequences of *H. costale*, *H. nelsoni*, and *C. virginica* (GenBank accession AF387122, U19538, and X60315,

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respectively) were aligned using the MacVector software package (Oxford Molecular Group) and regions unique to *H. costale* were identified. PCR primers SSO-A (5′-CACGACTTTGGCAGTTAGTTTTG-3′) and SSO-B (5′-CGAACAAGCGCTAGCAGTACAT-3′) and DNA probe SSO1318 (same sequence as SSO-B, 5′ end labeled with digoxigenin) were commercially synthesized (Genosys Biotechnologies).

PCR Amplification

PCR reaction mixtures contained reaction buffer (10 mM Tris, pH 8.3; 50 mM KCl; 1.5 mM MgCl; 10 μg/mL gelatin), 400 μg/mL bovine serum albumin, 25 pmoles each of SSO-A and SSO-B, 200 μM each of dATP, dCTP, dGTP, dTTP, 0.6 units AmpliTaq DNA polymerase (Perkin–Elmer), and template DNA in a total volume of 25 μL. The reaction mixtures were cycled in a GeneAmp PCR System 9600 thermal cycler (Perkin–Elmer) 35 times at 94°C for 30 sec, 59°C for 30 sec, and 72°C for 1.5 min with a final extension at 72°C for 5 min. PCR reaction mixtures and cycling conditions for *H. nelsoni* were identical, except the primers were MSX-A′ and MSX-B (Renault et al. 2000, Stokes et al. 1995a). An aliquot (10% of reaction volume) of each PCR reaction was checked for amplification product(s) by agarose gel electrophoresis and ethidium bromide staining.

PCR Specificity and Sensitivity

Primer specificity was tested in PCR reactions using cloned SSU rDNA from *H. costale*, *H. nelsoni*, *Haplosporidium louisiana* Sprague, and *Minchinia teredinis* Hillman, Ford, and Haskin, and genomic DNA from *Haplosporidium lusitanicum* Azevedo, *Minchinia chitonis* (Lankester), *Minchinia tapetis* (Vilela), and uninfected *C. virginica*. Preparation of the cloned SSU rDNAs were described previously (Stokes et al. 1995a). Hatchery-reared juvenile *C. virginica* were collected in July 1999, and genomic DNA was tested for the presence of *H. nelsoni* by PCR, as described previously (Stokes et al. 1995a). Limpets, *Helcion pellucidus*, were collected from Cap de La Hague, near Cherbourg, France in September 1998 and screened for the presence of *H. lusitanicum* spores. Chitons, *Lepidochitona cinereus*, were collected from Wembury Bay, near Plymouth, England in September 1996 and screened for the presence of *M. chitonis* spores. *Minchinia tapetis*-infected clams, *Ruditapes decussatus* (L.), collected from Vilalonga in the Ria de Arousa, Galicia, Spain, in 1997 were kindly supplied by Antonio Villalba. Spores were concentrated from infected tissues and DNA extractions from spores and from *C. virginica* were performed with mechanical grinding followed by detergent lysis, as described previously (Stokes et al. 1995b). Primer sensitivity to homologous target DNA was determined with ten-fold serial dilutions from 100 pg to 1 fg of cloned *H. costale* SSU rDNA.

Histology

Tissue samples were preserved in Davidson's AFA for at least 24 h. Fixed tissues were embedded in paraffin, sectioned 5–6-μm thick, and placed on positively charged slides (Fisher Scientific) for *in situ* hybridization or hematoxylin and eosin (H&E) staining. Tissue sections were kept in order as they were cut, and the consecutive sections were numbered on the slides. The microtome blade and forceps were cleaned with xylene between samples to prevent carry-over DNA contamination.

In Situ *Hybridization (ISH)*

Tissue sections for ISH were processed as described previously (Stokes & Burreson 1995), except hybridization solution contained 5 ng/μL SSO1318 DNA probe or 2 ng/μL MSX1347 DNA probe and the addition of Bismarck Brown Y counterstain after the nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate (BCIP) color development. Slides were washed with TE buffer (10mM Tris, pH 8.0; 1mM EDTA), then with dH_20 to stop the NBT/BCIP color development. Tissue sections were stained with 1% Bismarck Brown Y (Sigma Chemical) for 1 min, then rinsed three times with dH_20. The slides were coverslipped with GVA Mounting Solution (Zymed Laboratories) and examined by light microscopy. Negative control ISH consisted of dH_20 instead of DNA probe in the hybridization solution. Consecutive tissue sections of all samples were processed in the following order: section 1, stained with H&E; section 2, ISH with SSO1318; section 3, ISH with MSX1347, section 4, ISH with no probe.

DNA Probe Specificity

In situ hybridization with both DNA probes SSO1318 and MSX1347 were performed on four *C. virginica* that had been diagnosed by histological examination as infected only with *H. costale* (Table 1). The Virginia Marine Resources Commission (VIMS) Oyster Disease Archive reference numbers for these oysters, embedded in paraffin, are 177,822, 181,676, 181,677, and 196,774. All of these oysters were collected at Wachapreague, VA, on the sea side of Virginia's Eastern Shore, the type locality (Wood and Andrews 1962) for *H. costale*. To demonstrate the

TABLE 1.

Samples tested with in situ hybridizations using DNA probes SSO1318 and MSX1347.

Archive Sample Number	Collection Date	Diagnosis by Histological Examination	Diagnosis by *in situ* Hybridization
177,822	May 1988	*H. costale* heavy (spores present)	*H. costale* heavy; *H. nelsoni* heavy
181,676	May 1989	*H. costale* heavy (spores present)	*H. costale* heavy
181,677	May 1989	*H. costale* heavy (spores present)	*H. costale* heavy; *H. nelsoni* rare
196,774	October 1994	tentative *H. costale* heavy*	*H. costale* heavy; *H. nelsoni* light

* Diagnosis of 196,774 was uncertain. The infective agent appeared to be *H. costale*, however, such seasonality of an advanced infection was unprecedented.

Histological examination column indicates parasites identified in tissue sections and infection levels of original diagnoses. In situ hybridization column indicates parasites identified in tissue sections and infection levels with DNA probes. All samples were collected from the vicinity of Wachapreague, Virginia.

specificity of probe SSO1318, ISH was performed on sections of *C. virginica* tissues infected with *H. nelsoni* and *Perkinsus marinus* (Mackin, Owen, and Collier), of shipworm (*Teredo* sp.) tissue infected with *M. teredinis*, and of mud crab (*Panopeus* sp.) tissue infected with *H. louisiana*.

RESULTS

Specificity and Sensitivity of PCR Primers

The *H. costale* PCR primer pair SSO-A and SSO-B amplified a 557 bp region of the *H. costale* small subunit rDNA (Fig. 1A), targeting bases 784 to 1340 of that gene. The primers did not amplify DNA from oyster or from the haplosporidans *H. nelsoni*, *H. louisiana*, *H. lusitanicum*, *M. teredinis*, *M. chitonis*, or *M. tapetis* (Fig. 1A). The PCR product was readily detected after amplification of 100 fg to 100 pg of cloned *H. costale* SSU rDNA; 10 fg of template DNA was amplified, but the product band was very faint in the agarose gel (Fig. 1B).

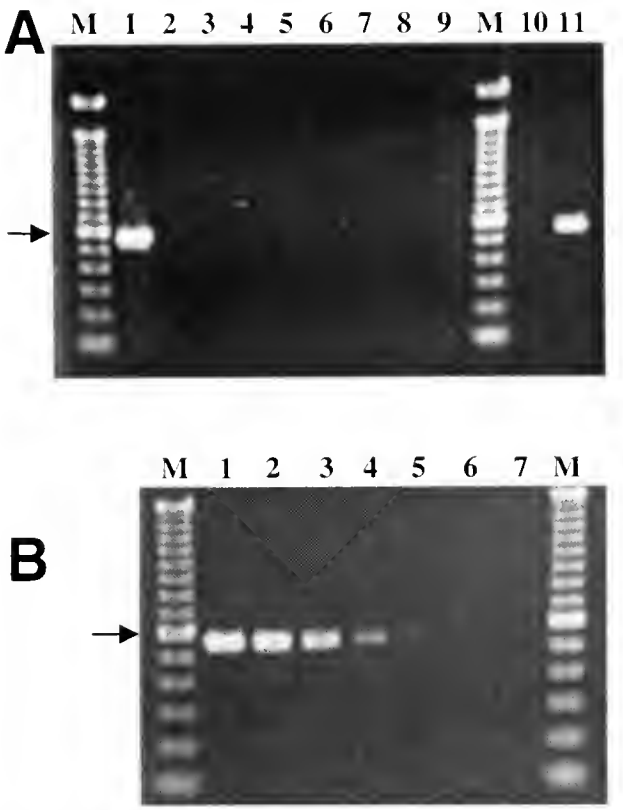

Figure 1. Specificity and sensitivity of *Haplosporidium costale* PCR primers. (A) Specificity. Lanes M, 100 bp ladder size marker, arrow indicates 600 bp; lane 1, cloned *H. costale* SSU rDNA; lane 2, cloned *H. nelsoni* SSU rDNA; lane 3, *H. lusitanicum* genomic DNA; lane 4, cloned *H. louisiana* SSU rDNA; lane 5, cloned *Minchinia teredinis* SSU rDNA; lane 6, *M. chitonis* genomic DNA; lane 7, *M. tapetis* genomic DNA; lane 8, uninfected *Crassostrea virginica* genomic DNA; lane 9, no DNA control; lane 10, *H. nelsoni*-infected *C. virginica* genomic DNA; lane 11, *H. costale*-infected *C. virginica* genomic DNA. (B) Sensitivity. PCR amplification products using *H. costale* primers SSO-A and SSO-B against serial dilutions of cloned *H. costale* SSU rDNA template. Lanes M: 100 bp ladder size marker, arrow indicates 600 bp; lane 1: 100 pg template DNA; lane 2: 10 pg; lane 3: 1 pg; lane 4: 100 fg; lane 5: 10 fg; lane 6: 1 fg; lane 7: no DNA control.

Specificity of DNA Probe

One of the candidate *H. costale* probes, designated SSO1318, was found to be sensitive and specific for *H. costale* in *in situ* hybridizations of tissue sections. Optimal hybridization required 5 ng/μL SSO1318 and incubation at 42°C. The SSO probe readily detected *H. costale* plasmodia and immature spores in sporocysts in ISH of oyster tissue with virtually no background, as indicated by the cells that stained dark purple to black (Fig. 2). DNA probe SSO1318 did not hybridize with oyster tissue (*C. virginica*), the oyster pathogen *P. marinus*, or the haplosporidans *H. nelsoni*, *H. louisiana*, and *Minchinia teredinis* (Fig. 3).

Differential Diagnosis using DNA Probes

Four oysters previously diagnosed by routine histological examination of H&E-stained paraffin sections as infected with *H. costale*, but not *H. nelsoni* (Table 1) were subjected to ISH using separate DNA probes for *H. costale* and *H. nelsoni*. Plasmodia and immature spores in tissues of all four oysters hybridized with the SSO probe (Figs. 4–6), thus supporting the histological diagnoses. However, some plasmodia in three of the four oysters did not hybridize with the *H. costale* probe, but instead hybridized with the *H. nelsoni* probe (Figs. 4–6). These mixed infections of *H. costale* and *H. nelsoni* were not distinguishable nor detectable by histological examination in part because only plasmodial stages of *H. nelsoni* were present, but they were readily apparent by *in situ* hybridization (ISH) using the species-specific DNA probes. Even a light infection of *H. nelsoni* plasmodia, scattered among a heavy infection of *H. costale* was easily detected using a DNA probe (Fig. 4E). In addition, the *H. costale* probe enabled discrimination of early and maturing plasmodia, the latter, which have vacuolated cytoplasm (Wood & Andrews 1962). The vacuoles within the stained plasmodia are easily seen at low power in ISH with the *H. costale* probe (Fig. 5B). *Haplosporidium nelsoni* plasmodia were found in both connective tissue and epithelia (Figs. 4E, F; 5C; 6C); *H. costale* plasmodia were located throughout the connective tissue but not in the epithelium of the four oysters examined (Figs. 4C, D; 6B).

One oyster collected in October 1994 seemed to be infected with *H. costale* based on the presence of plasmodia and appropriately sized immature spores within sporocysts scattered throughout the connective tissue as determined by stained paraffin sections (Table 1). However, the diagnosis was recorded as tentative, because advanced infections of *H. costale* were known only from April to June (Andrews & Castagna 1978) and none had ever been reported from the fall season. *In situ* hybridizations using both *H. costale* and *H. nelsoni* DNA probes confirmed a mixed infection of the two parasites (Fig. 6), thus documenting unprecedented timing of an advanced *H. costale* infection. Plasmodia and sporocysts of *H. costale* were abundant in connective tissue (Fig. 6B), but not in epithelium; plasmodia of *H. nelsoni* occurred in epithelium (Fig. 6C) but not in connective tissue.

DISCUSSION

The PCR primers SSO-A and SSO-B and the DNA probe SSO1318 were sensitive and specific for the target organism, *H. costale*. Another set of PCR primers for *H. costale* was previously reported by Ko et al. (1995); however, we chose to target a different region of the SSU rDNA. The two regions targeted by the probe and primers described here are highly variable within the phylum Haplosporidia, accessible for probe hybridization and have

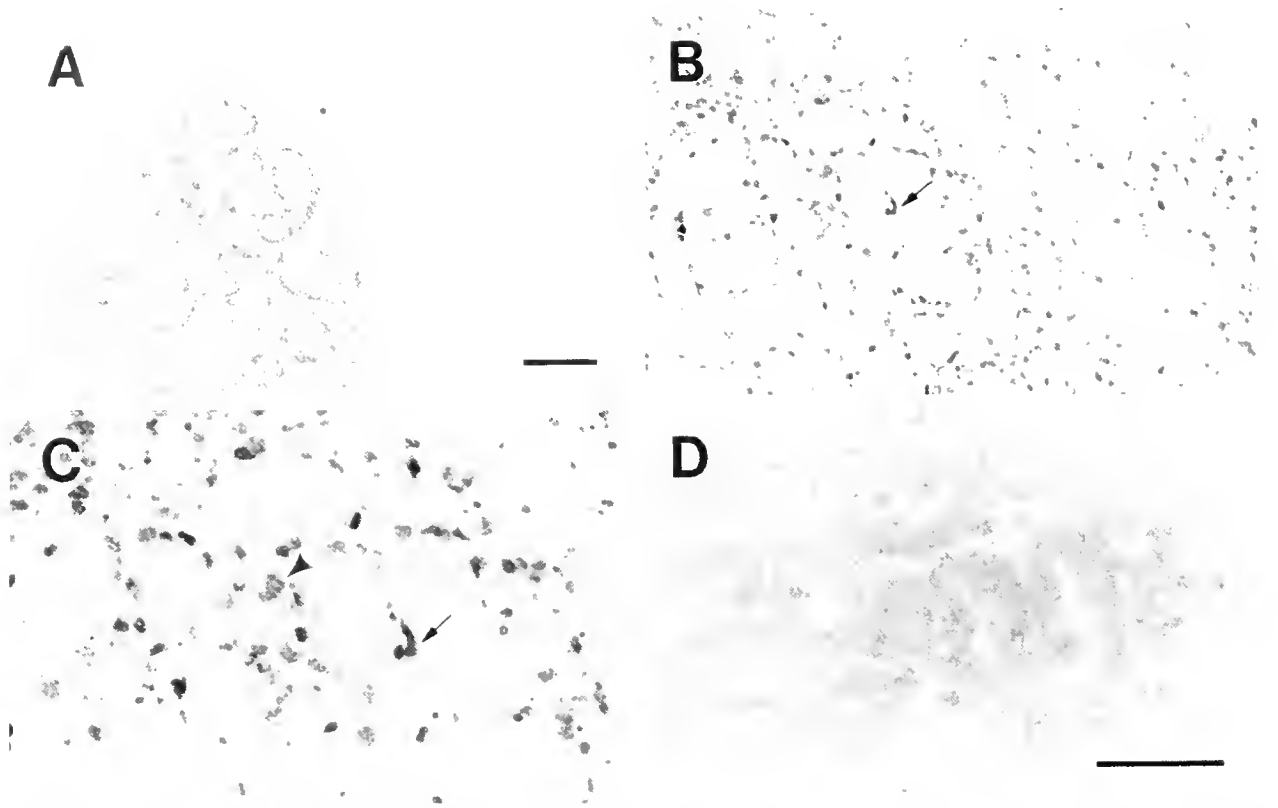

Figure 2. Consecutive histological sections of *H. costale*-infected *C. virginica* tissue (#181,676) showing plasmodia and sporocysts containing immature spores in the connective tissue. (A) Hematoxylin and eosin (H&E) stain. Bar = 100 µm and also applies to B. (B) *In situ* hybridization (ISH) with *H. costale* DNA probe. Arrow points to plasmodium enlarged in C. (C) ISH at higher magnification, arrow points to same plasmodium indicated in B. (D) ISH with *H. nelsoni* DNA probe. Bar = 100 µm and also applies to C.

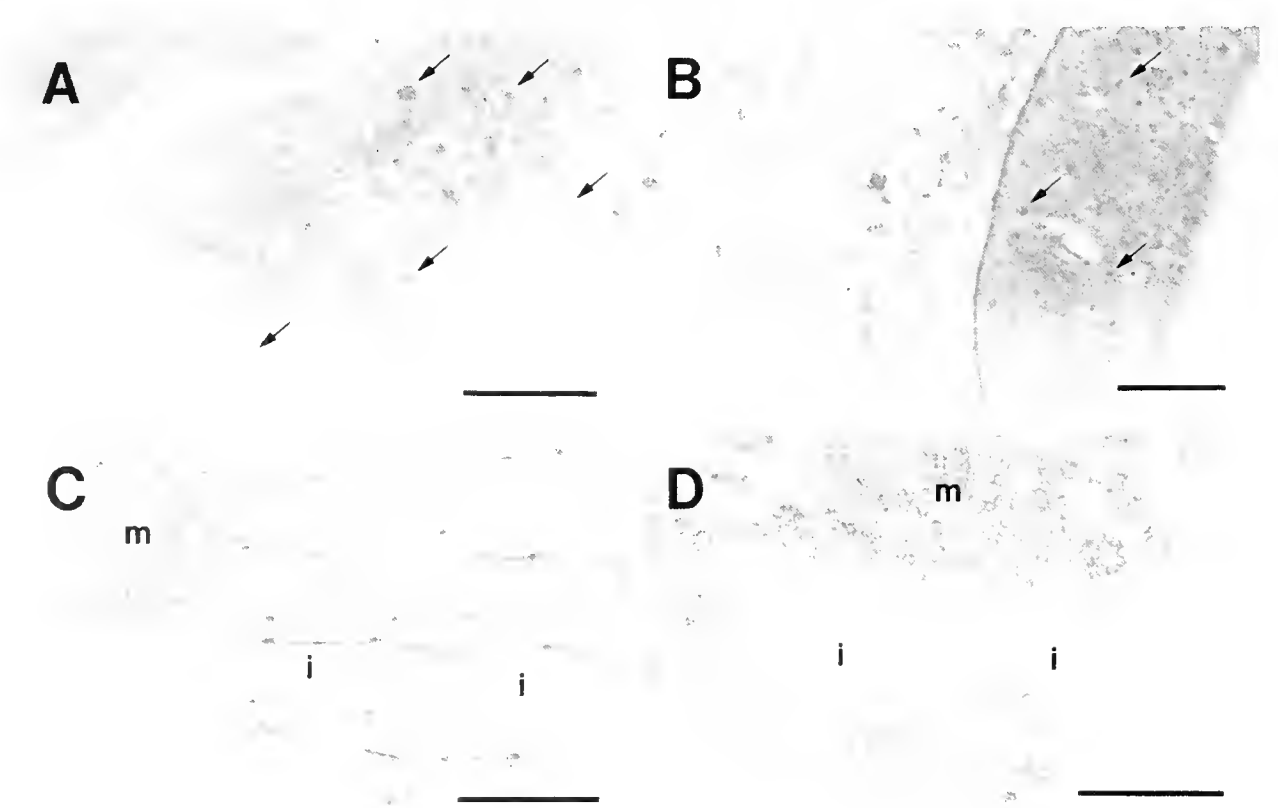

Figure 3. Lack of *in situ* hybridization (ISH) of various other parasites in histological sections demonstrating specificity of the *H. costale* DNA probe. (A) *H. nelsoni*-infected *C. virginica* tissue, arrows indicate some of the plasmodia present. Bar = 100µm. (B) *Perkinsus marinus*-infected *C. virginica* tissue, arrows indicate some of the cells present in the epithelium. Bar = 60 µm. (C) *M. teredinis*-infected *Teredo* sp. tissue, with immature (i) and mature (m) spores. Bar = 100 µm. (D) *H. louisiana*-infected *Panopeus* sp. tissue, with immature (i) and mature (m) spores. Bar = 100 µm.

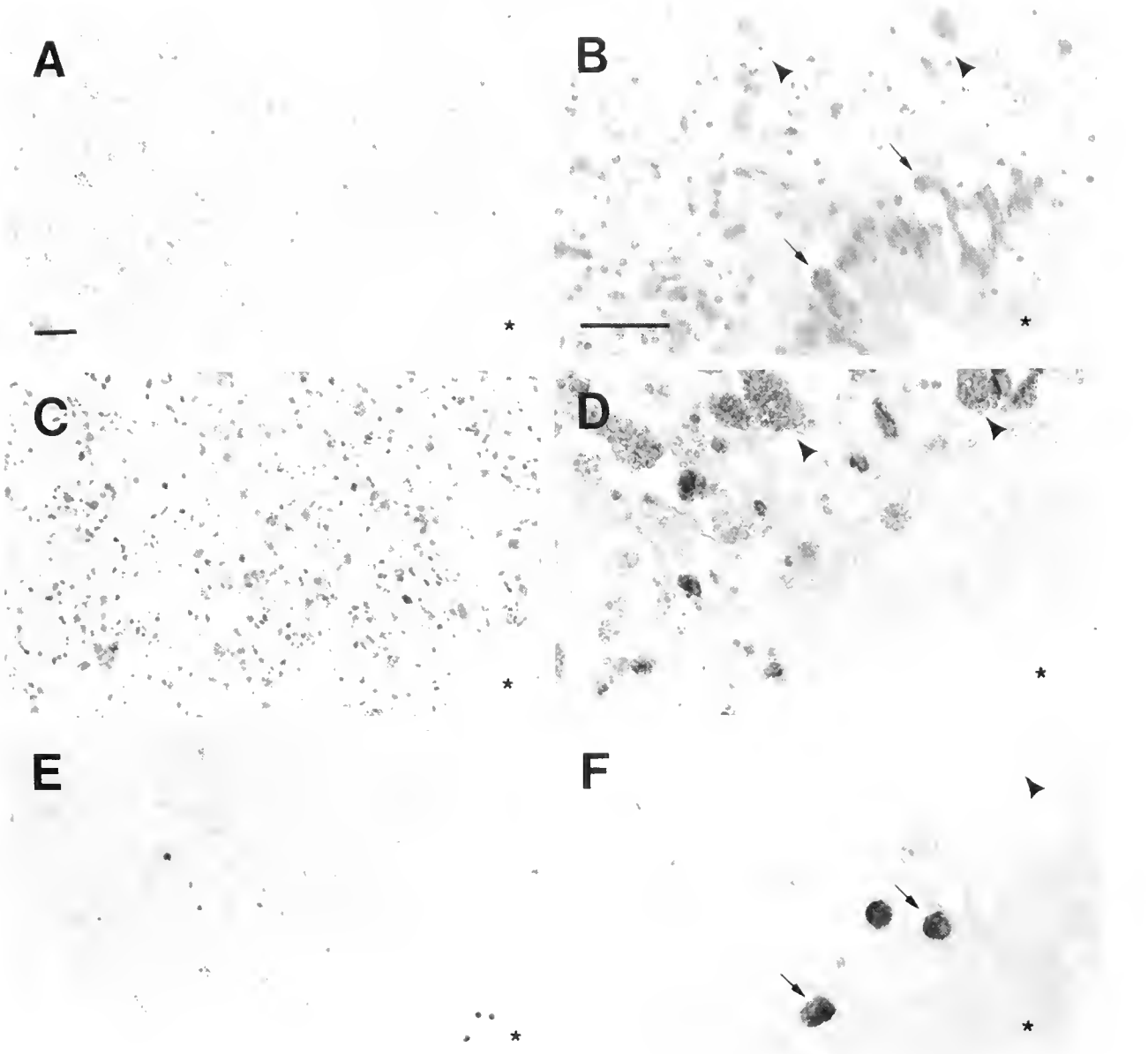

Figure 4. *In situ* **hybridization (ISH) of consecutive histological sections of oyster tissue (#181,677) collected in May 1989 from Virginia's Eastern Shore with a mixed haplosporidan infection. A, C, E all show the same area; B, D, F are higher magnifications of A, C, and E, respectively. Asterisk in lower right of each figure indicates the same epithelial lobe. (A) Hematoxylin and eosin (H&E) stain. Bar = 60 µm and also applies to C and E. (B) Hematoxylin and eosin (H&E) stain showing** *H. nelsoni* **plasmodia (arrows) in the epithelium and some of the** *H. costale* **sporocysts (arrowheads) in the connective tissue. Bar = 30 µm and also applies to D and F. (C, D) ISH with** *H. costale* **DNA probe of same region in A and B showing positive reaction with** *H. costale*, **but not** *H. nelsoni*. **Arrowheads in D point to same** *H. costale* **sporocysts as in B. (E, F) ISH with** *H. nelsoni* **DNA probe of same region in A and B showing positive reaction with** *H. nelsoni* **plasmodia, but not with** *H. costale* **sporocysts. Note ability of DNA probe to identify rare** *H. nelsoni* **plasmodia in a heavy** *H. costale* **infection. Arrows in F indicate hybridization of** *H. nelsoni* **plasmodia in the epithelium as shown in B; arrowhead in F indicates lack of reaction with** *H. costale* **sporocyst shown in B and D.**

been used successfully for *H. nelsoni*-specific diagnostics (Stokes & Burreson 1995, Stokes et al. 1995a). The *H. costale* probe hybridized with *H. costale* plasmodia and immature spores, but not with mature spores, the same hybridization pattern as with the MSX probe with *H. nelsoni* (Stokes & Burreson 1995). In ISH of oyster samples from France, the SSO1318 probe did not hybridize with the Pacific oyster *C. gigas* nor with a haplosporidian infecting that host (Renault et al. 2000).

Mixed infections of *H. costale* and *H. nelsoni* that have not advanced to sporulation can now be diagnosed with confidence using these new tools. The plasmodia that hybridized with the *H. nelsoni* probe were not the same plasmodia that hybridized with the *H. costale* probe; although, these plasmodia were indistinguishable by traditional histological examination of stained tissue sections. The mixed parasite infections described here were originally diagnosed as being only *H. costale*. This diagnosis was undoubtedly made because of the preponderance of *H. costale* plasmodia and immature spores as compared to the relatively light infections of *H. nelsoni* and also because spores of *H. nelsoni* were absent. Couch (1967) reported finding mixed infections of *H. cos-*

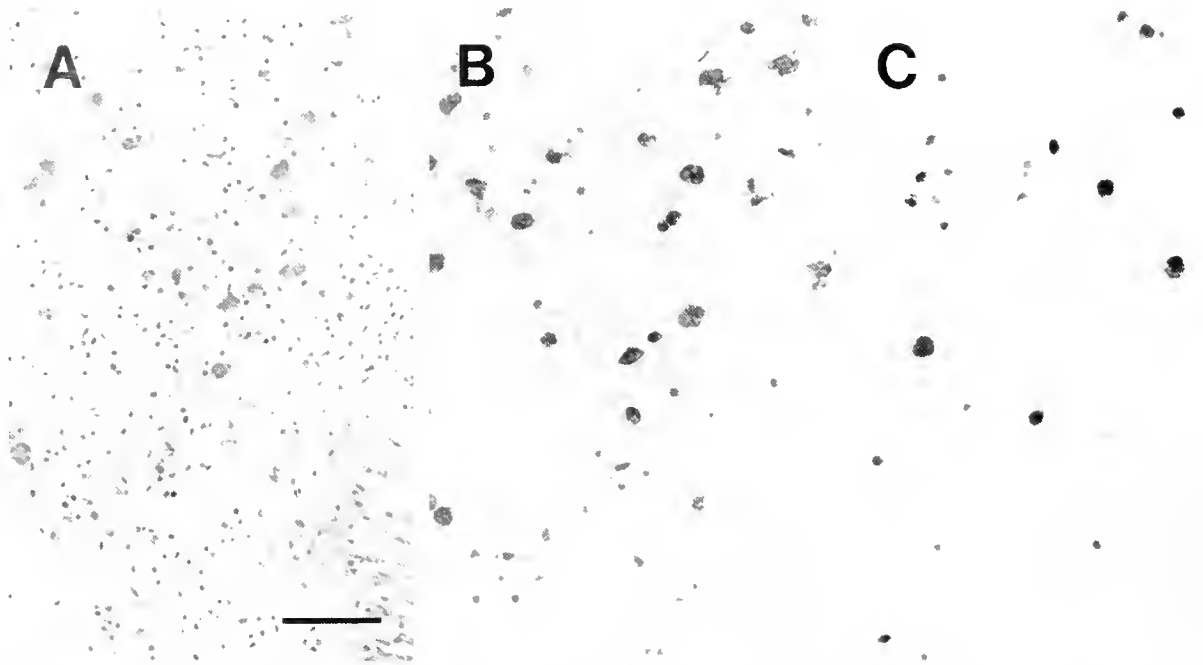

Figure 5. *In situ* **hybridization (ISH) of consecutive histological sections of oyster (#177,822) collected in May 1988 from Virginia's Eastern shore with mixed haplosporidan infection illustrating ease of plasmodia differentiation with DNA probes. (A) Hematoxylin and eosin (H&E) stain. Bar = 50 µm and applies to B and C. (B) ISH with** *H. costale* **DNA probe of same area shown in A. (C) ISH with** *H. nelsoni* **DNA probe of same area shown in A and B.**

tale and *H. nelsoni*, but they were based on the presence of spores of both species. In oysters tested to date with DNA probes, we have not observed *H. costale* plasmodia in the epithelium.

The inability to distinguish nonsporulating mixed haplosporidian infections by traditional histological examination may have skewed epizootiology data for high salinity regions in the past. It is possible that *H. nelsoni* has been more common in Virginia oysters in high salinity than previously reported. If so, this may raise questions about past disease data and oyster mortality attributed to *H. costale*.

Results of diagnoses using DNA probes have revealed an unprecedented seasonality of *H. costale* infections. The original diagnosis of *H. costale* infection in oyster #196,774 in October 1994 was uncertain. The plasmodia and immature spores throughout the connective tissue looked like *H. costale*, but the timing of this advanced infection was unusual. Epizootiological studies of *H. costale* had established the annual infection cycle as quite predictable. Clinical infections appear in the spring, as early as March, with sporulation and oyster mortality primarily in May and June. New infections occur before August 1st but remain subclinical

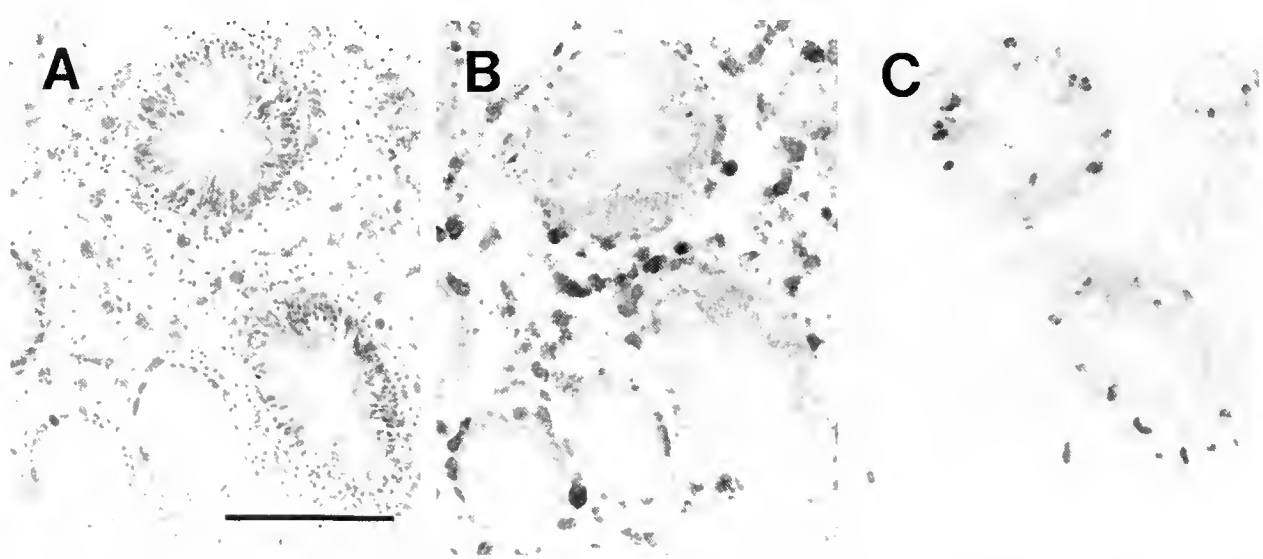

Figure 6. *In situ* **hybridization (ISH) of consecutive histological sections of oyster (#196,744) collected in October 1994 from Virginia's Eastern Shore with mixed haplosporidan infection. (A) Hematoxylin and eosin (H&E) stain. Bar = 100 µm and applies to B and C. (B) ISH with** *H. costale* **DNA probe of same area shown in A. Note** *H. costale* **plasmodia in connective tissue, but not in epithelium. (C) ISH with** *H. nelsoni* **DNA probe of same area shown in A and B. Note** *H. nelsoni* **plasmodia in epithelium, but not in connective tissue.**

until the following spring (Couch & Rosenfield 1968, Andrews & Castagna 1978, Andrews 1988). Andrews and Castagna (1978) reported that numerous samples of seaside Virginia oysters from summer through winter revealed no *H. costale* infections. Diagnosis by DNA probes of oyster 196,774 confirmed the *H. costale* diagnosis, as about 80% of the plasmodia hybridized with the SSO1318 probe, but also revealed it as a mixed infection, because about 20% of the plasmodia hybridized with the MSX1347 probe. This *H. costale* infection, where the parasite's identity was confirmed by DNA-based diagnostics, did not meet historical criteria for SSO disease suggesting that the seasonality and epizootiology of this pathogen must be re-examined.

ACKNOWLEDGMENTS

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ANALYSES OF GONADAL CYCLING BY OYSTER BROODSTOCK, *CRASSOSTREA VIRGINICA* (GMELIN), IN LOUISIANA

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ABSTRACT Oysters held near-shore in Caminada Bay, Louisiana during the summer, exhibit hypertrophic gonads with prominent genital canals beneath transparent mantle tissue about four weeks post-hatchery spawning, indicating recycling. Broodstock (N = 200) were analyzed histologically over a two-year period to document such gametogenesis, using Gonad/Body Ratios (GBR) and developmental stages. Ten oysters were randomly selected from a broodstock pool prior to each spawning attempt, and monthly during the winter-spring. As expected, the mean GBR before successful spawning attempts was significantly greater ($P \leq 0.05$) than the mean GBR before unsuccessful attempts. A dramatic drop in the percent occurrence of the advanced spawning and regression stage from May to June, a >40% spawning stage occurrence from May to October, and fluctuations in the percent occurrence of early and late developmental stages during the summer months illustrates gonadal recycling.

KEY WORDS: *Crassostrea virginica*, oyster, gonad, development, cycling, Louisiana

INTRODUCTION

Broodstock gonadal condition is fundamental to consistent production of oyster larvae (Lannan et al. 1980) and successful triploid induction (Downing & Allen 1987). Although temperature requirements for gonadal development and spawning have been reported for the southern variety of the eastern oyster (*C. virginica*) (Hopkins 1931, Hopkins et al. 1953, Loosanoff 1969, Hayes and Menzel 1981, Gauthier and Soniat 1989) documentation of the relationship between gametogenesis and induced spawning in the Gulf of Mexico region is limited. This is of particular interest, since oysters from southern populations are multiple spawners indicated by repeated gametogenic development throughout the spawning season (Ingle 1951, personal hatchery observations). An understanding of gonadal redevelopment is critical for collection of large numbers of viable eggs to produce commercial-scale broods, especially triploids (Allen et al. 1989)

Oysters held near-shore at the hatchery on Caminada Bay, Louisiana redevelop gonad and can be spawned repeatedly in the summer. Hypertrophic gonads with prominent genital canals beneath transparent mantle tissue can occur about 4 weeks post-spawning. Such gonadal activity stimulated interest in histological examination of gonadal cycling in the hatchery's broodstock. The purpose of this study was to document oyster broodstock gametogenesis, including winter development leading up to the spawning season, redevelopment (or recycling) between spawnings, and to determine the relationship between gonadal condition and spawning success.

MATERIALS AND METHODS

A broodstock of oysters was collected from oyster reefs in Louisiana and maintained in containers near-shore at the Sea Grant Grand Isle Bivalve Hatchery (29°15′12″N, 90°03′26″W) on Caminada Bay. Ten oysters were randomly selected over a two-year period for histological analyses when brought to the hatchery, prior to each spawning attempt, and bimonthly from the remaining broodstock during the winter-spring.

A 4–5 mm cross-section of each oyster was removed just posterior to the labial palp-gill junction and preserved in Davidson's fixative for histological processing (Howard & Smith 1983). A 4 µm section was obtained approximately 1,000 µm from the junction, mounted and stained with Gill's hematoxylin and eosin. This allowed the use of a standard cross section from each oyster for comparison. The sections were characterized by two H-shaped structures (large appendix of the stomach cecum) ventrally located in the histological sections as described by Morales-Alamo and Mann (1989).

Qualitative Description

Individual sections were microscopically examined to determine the sex and stage of gamete development for each oyster. Classifications included Early Development, Late Development, Spawning, and Advanced Spawning and Regression (ASR) after Kennedy and Krantz (1982).

Quantitative Analyses

Gonad/Body Ratios (GBR) were generated from histological sections similar to Kennedy and Battle (1964) using equidistant transects across each section to determine the gonadal width relative to body width (Fig. 1). Only transects 3 to 8 were used. However, preliminary statistical comparison of mean GBR's among transects indicated that transects from the dorsal and ventral regions of the gonads were different from the rest, likely a result of the shape of the right and left gonads (Fig. 1). Histological sections of oysters (N = 200) were analyzed to determine GBR differences among developmental stages and spawning attempts.

GBR was also measured in groups of oysters used during normal hatchery operation to determine how GBR varied between spawning and non-spawning populations. During each spawning attempt, approximately 150 oysters were removed from near-shore containers, 10 were randomly selected for histological analysis and the remainder were placed in a spawning table and exposed to ambient (30°–35°C) seawater for about 30 minutes. If necessary, the broodstock were then exposed to 18°C for approximately 1 hour, then re-exposed to ambient conditions for approximately 2 hours to stimulate spawning. If temperature stimulation did not

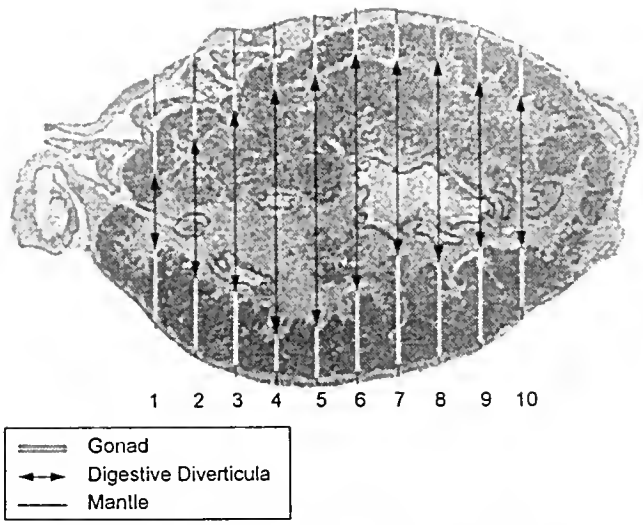

Figure 1. Image of histological transverse section through the mid-body region of an oyster. Measurements from transects 3 to 8 were used in determining the gonad/body ratio.

lead to spawning, the broodstock were exposed to a sperm suspension. Successful spawning was noted when a majority of the oysters had spawned, while unsuccessful spawning was characterized by little or no spawning activity.

The GBR's were analyzed using analysis of variance (ANOVA) with a two-way fixed factorial model (SAS Institute 1991) to test the difference among spawning attempts. The model included GBR as the dependent variable, spawning result as a fixed effect, and developmental stage and its interaction with the spawning result as random effects. The GBR's met the assumptions of normality and variance homogeneity after angular transformation (Dowdy and Wearden 1991). Tukey's Honestly Significant Difference Procedure was used to test the difference (α = 0.05) among developmental stage and spawning result.

RESULTS

Quantitative Analyses

As expected, all developmental stages had significantly different mean GBR's ($P \leq 0.05$)(Table 1) and the mean GBR during successful spawning attempts was significantly greater (mean = 0.51, $P \leq 0.05$) than the mean GBR during unsuccessful attempts (mean = 0.45)(Table 2).

TABLE 1.

Results of analysis of variance: comparing mean gonad/body ratio of *Crassostrea virginica* by developmental stage.

Developmental Stage	Ratio* Mean	SD	Comparisons**
Early Development	0.31	0.14	A
Later Development	0.46	0.12	B
Spawning	0.52	0.13	C
Advanced Spawning & Regression	0.38	0.15	D

* Ratio = Arsin (√ (Gonad Width/Body Width)).
** Tukey's Honestly Significant Difference (α = 0.05).
SD = Standard Deviation.

TABLE 2.

Results of analysis of variance: comparing mean gonad/body ratios of *Crassostrea virginica* by spawning result.

Spawning Result	Ratio* Mean	SD	Comparisons**
Successful	0.51	0.10	A
Unsuccessful	0.45	0.12	B

* Ratio = Arcsin (√ (Gonad Width/Body Width)).
** Tukey's Honestly Significant Difference (α = 0.05).
SD = Standard Deviation.

Qualitative Analyses

After evaluating the histological sections for developmental stage (Kennedy and Krantz 1982), it became apparent that oyster gonads were recycling. Histological sections representing Early Development (Fig. 2, A & B), Late Development (Fig. 3, A & B), Spawning (Fig. 4, A & B) and Advanced Spawning-Regression (ASR)(Fig. 5, A & B) where noticeably different than sections from recycling (Fig. 6, A & B). ASR gonads were typically atretic, with significant amounts of cellular debris and amoebocytes in

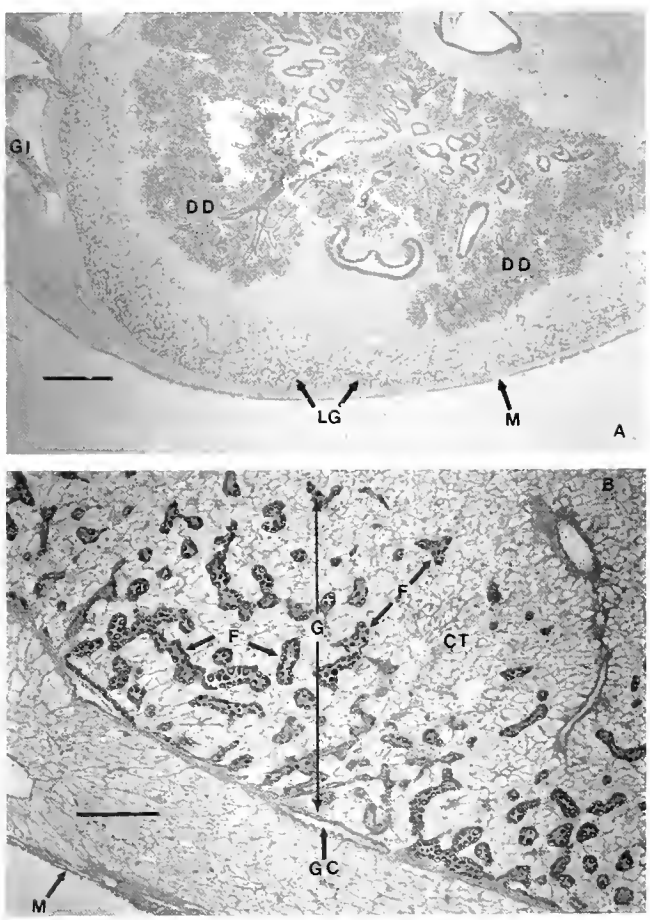

Figure 2. A & B. Photomicrographs if histological sections showing early gonadal development (March) of *C. virginica*, posterior-ventral region (A: bar = 200μ; B: bar = 30μ). LG = left gonad, DD = digestive diverticula, M = mantle, GI = gill, G = gonad, F = follicles, CT = connective tissue, CG = genital canal.

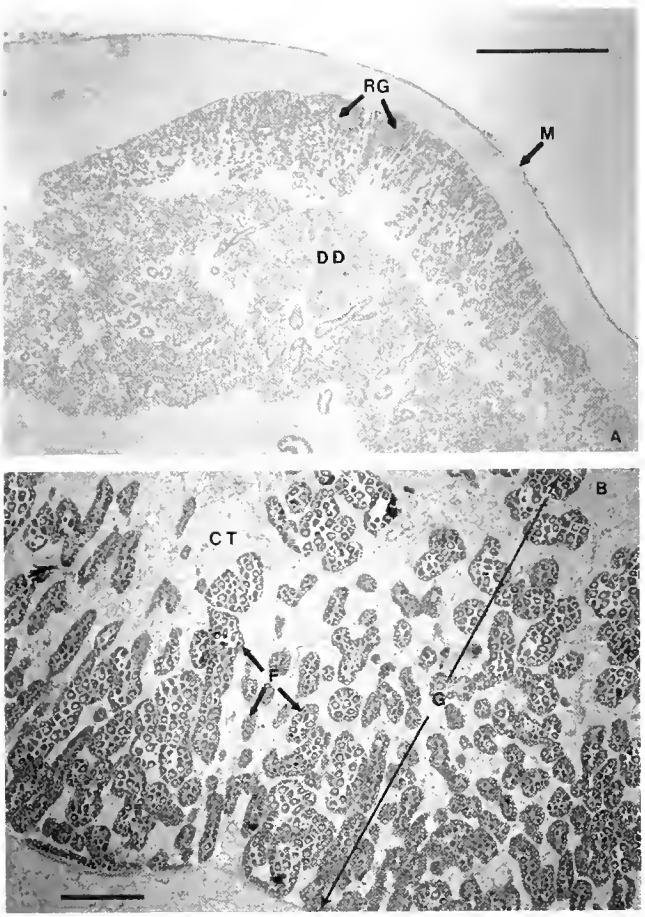

Figure 3. A & B. Photomicrographs of histological sections showing later gonadal development (April) of *C. virginica*,posterior-dorsal region (A: bar = 200μ; B: bar = 30μ). RG = right gonad, DD = digestive diverticula, M = mantle, G = gonad, F = follicles, CT = connective tissue.

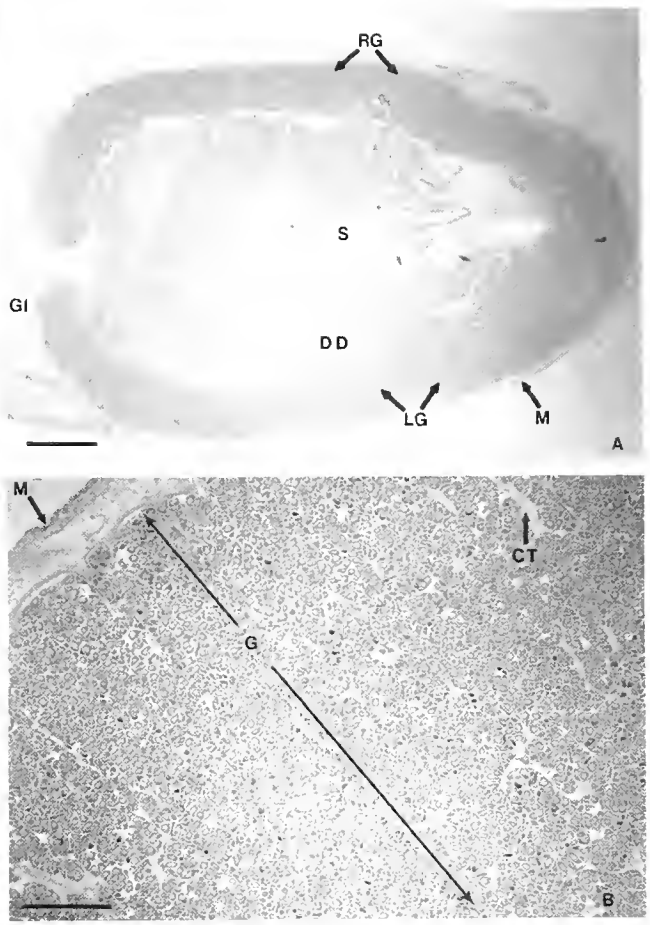

Figure 4. A & B. Photomicrographs of histological sections showing spawning gonadal development (June) of *C. virginica*. (A: bar = 200μ; B: bar = 30μ, posterior-dorsal region). RG = right gonad, LG = left gonad, DD = digestive diverticula, M = mantle, GI = gill, S = stomach, G = gonad, CT = connective tissue.

nearly empty follicles and surrounding connective tissue. In contrast, recycling gonads were characterized by proliferation of developing follicles typically found in Early and Late Development sections (i.e., enlarged germinal cells and young pendant primary oocytes in females [Kennedy & Battle 1964]).

Figure 7 illustrates the gametogenic cycle produced by the descriptive stage characterization conducted during this study. A high percentage of the oysters examined were in Early Development at the beginning of the year; this stage decreased from May to November, rose during September, and increased again in December. A short occurrence of Late Development in the spring during the sharp rise in the occurrence of the Spawning stage shows how oysters held in the near-shore waters of Grand Isle rapidly developed toward spawning condition. The percent of oysters in Later Development fluctuated during the summer. The occurrence of ASR reached 60% during April to May, decreased to approximately 25% during May to June, remained stable during the summer, and increased dramatically beginning in September to October.

DISCUSSION

Quantitative Results

The difference between the mean GBR of oysters that spawned and those that did not was as expected (Table 2). Oysters in spawning condition should have higher GBR's than non-spawning oysters. Follicles anastomose through the surrounding connective tissue during gonadal development (Figs. 2–6) increasing the mean GBR. The creamy-white appearance of a sexually mature oyster is due to the hypertrophy of the gonad (Kennedy & Battle 1964; Fig. 1).

Fecund oysters are prolific spawners. Such spawning activity is reflected in the mean GBR's in Table 1, which depict gonadal attrition between spawning (0.52) and advanced spawning and regression (0.38). Hormonal control (Morse et al. 1978), genetics (Lannan 1980) and food availability to broodstock are examples of the many sources of variation in obtaining successful oyster spawning.

Qualitative Results

The occurrence of oysters in Spawning stage in Figure 7 depicts an expected curve of over 60% occurrence during May to October, with reductions in the winter and spring. A similar autumn gonadal state has also been documented in Louisiana (Gauthier & Soniat 1989).

One would think that a line depicting an ASR gonadal stage would sharply increase from June to December, but this is not evident in Figure 7. The line depicts the highest ASR occurrence

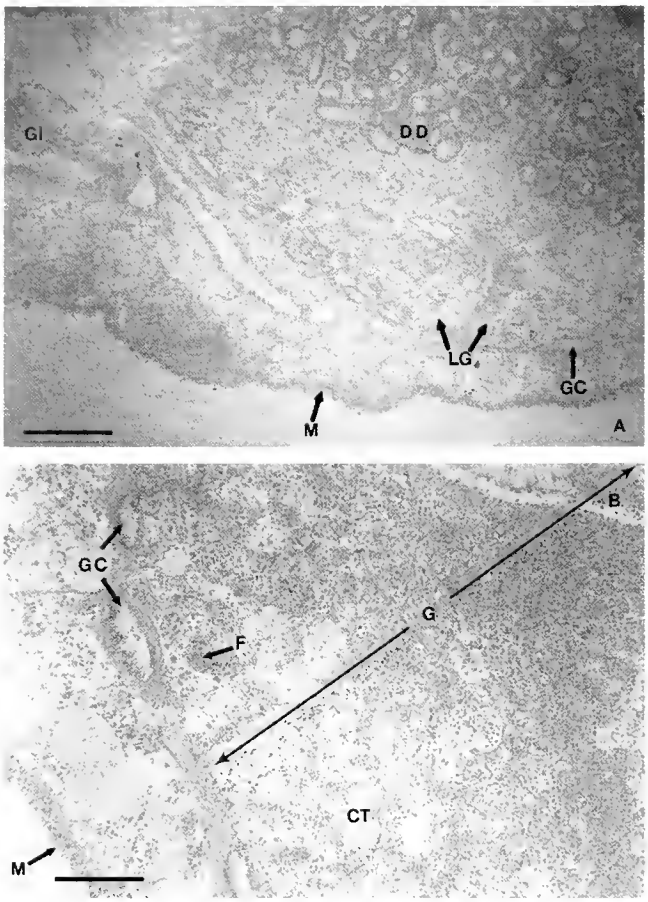

Figure 5. A & B. Photomicrographs of histological sections showing advanced spawning and regression gonadal development (August) of *C. virginica*, posterior-ventral region (A: bar = 200μ; B: bar = 30μ). LG = left gonad, DD = digestive diverticula, GC = genital canal, M = mantle, GI = gill, G = gonad, CT = connective tissue, F = follicle.

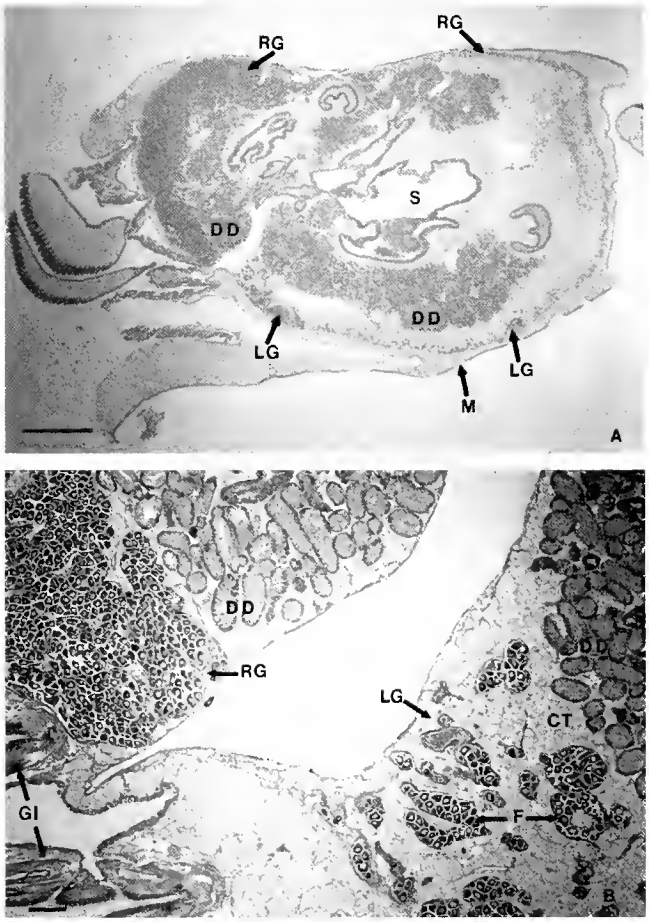

Figure 6. A & B. Photomicrographs of histological sections showing gonadal recycling (October) of *C. virginica* (A: bar = 200μ; B: bar = 30μ; ventral region). RG = right gonad, LG = left gonad, DD = digestive diverticula, S = stomach, M = mantle, GI = gill, CT = connective tissue, F = follicle.

in May (60%) and December (80%) with lower occurrences (24%–39%) during June to July.

The dramatic drop in ASR occurrence and the rather steady occurrence of the Spawning stage, as well as fluctuations in the percent occurrence of Early and Later Developmental stages during the summer months, illustrates recycling during June to October. This may or may not be evidence of spawning in the wild since the samples were taken from suspended broodstock coerced to spawn at the hatchery during the study.

Nevertheless, comparison of Figures 2 to 5 with Figures 6, 8 and 10 shows how gonadal recycling is occurring in the broodstock held at the Grand Isle hatchery. The follicles of the left gonad depicted in Figure 6 (B) and those depicted in Figure 8 (B) are similar to those found during Early and Later Development (Figs. 3 and 4). Atresia is evident in the dorsal region of the right and left gonads in Figure 6 (A) and in the nearby connective tissue and follicles in Figures 6 (B) and 8 (B). This atresia is similar to that found in Advanced Spawning and Regression (Fig. 5). The right gonad in Figure 6, Figure 8 (A), and Figure 9 are similar to the gonads in Spawning stage oysters in Figure 6, with no noticeable atresia nor reduction in oocyte organization within the follicles (Kennedy & Krantz 1982).

The description of ASR by Kennedy and Krantz (1982) included the occurrence of early gametogenic stages remaining on the follicle walls and the reappearance of connective tissue in the interfollicular areas with invading phagocytic cells. The use of an additional developmental stage, recycling, including the presence of early and later development characteristics plus the presence of atresia, is recommended to improve the characterization of gonadal development in Louisiana.

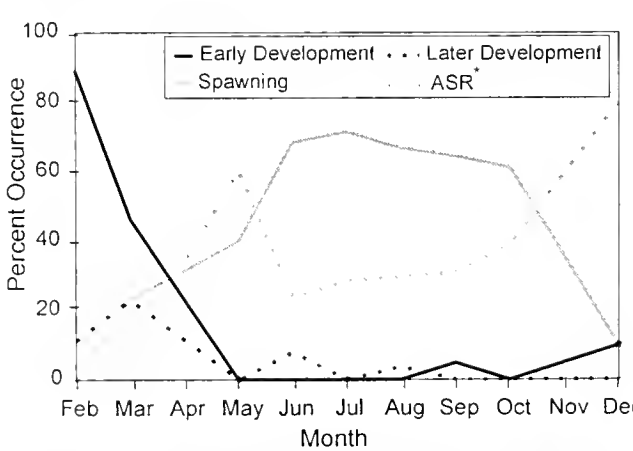

Figure 7. Percent occurrence of gonad development stage of *C. virginica* by month. * denotes advanced spawning and regression.

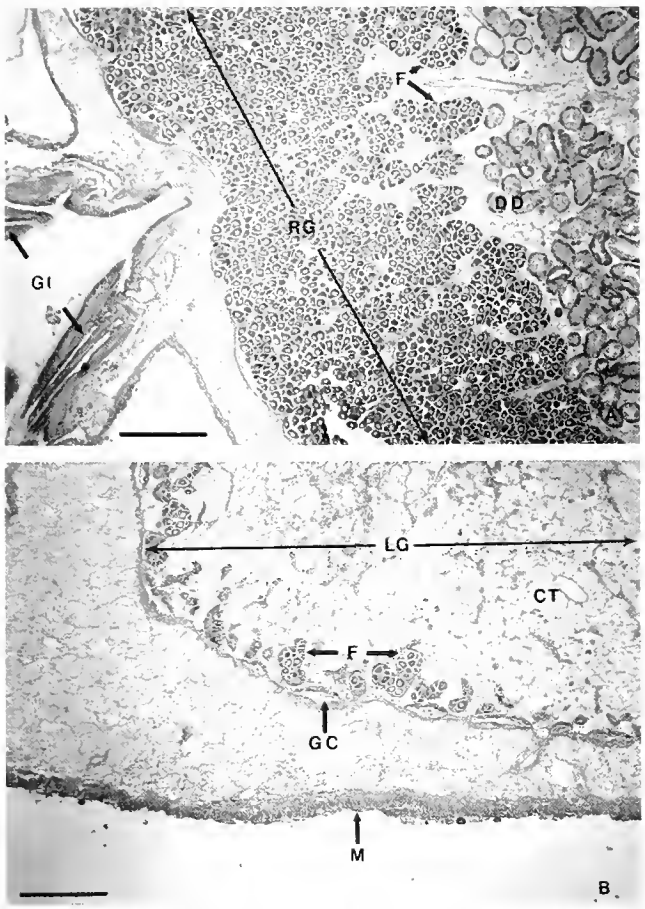

Figure 8. A & B. Photomicrographs of histological sections showing gonadal recycling (August) of *C. virginica* (A: bar = 200μ; B: = 200μ, dorsal region). RG = right gonad, LG = left gonad, DD = digestive diverticula, M = mantle, GI = gill, CT = connective tissue, S = stomach, GC = genital canal.

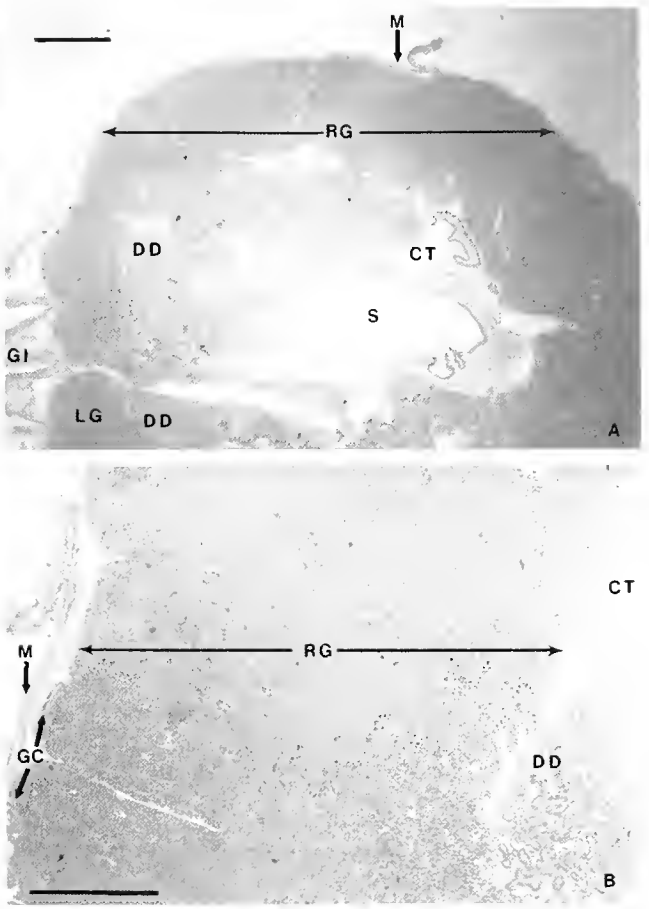

Figure 9. A & B. Photomicrographs of histological sections showing gonadal recycling (August) of *C. virginica* (A: bar = 200μ; B: bar = 200μ, dorsal region). RG = right gonad, LF = left gonad, DD = digestive diverticula, M = mantle, GI = gill, CT = connective tissue, S = stomach, GC = genital canal.

Histological sectioning is expensive and, therefore, not a valuable tool for evaluating hatchery broodstock. Gonadal recycling of broodstock held near-shore is visually apparent, however, by the observance of transparent mantle tissue occurring with a spotty or hypotrophic gonad during early recycling, followed by a full or hypertrophic gonad after complete recycling.

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A COMPARATIVE FIELD STUDY OF *CRASSOSTREA ARIAKENSIS* (FUJITA 1913) AND *CRASSOSTREA VIRGINICA* (GMELIN 1791) IN RELATION TO SALINITY IN VIRGINIA

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ABSTRACT We examined survival, growth, and disease susceptibility of triploid *Crassostrea ariakensis* (= *rivularis*) and compared results with that of diploid *Crassostrea virginica*. Two hundred and fifty oysters (age = 2 yr, mean shell height = 60–64 mm) of each species were deployed at duplicate sites, (Chesapeake Bay, and the Atlantic Coast of Virginia) within low, medium, and high salinity regimes respectively (< 15‰, 15–25‰, > 25‰). Over the course of the study, from June 1998 to September 1999, *C. virginica* exhibited low survival, modest growth and high disease susceptibility. In contrast, *C. ariakensis* exhibited high survival, high growth rate, and low disease susceptibility. At low salinity sites, final mean cumulative mortality of *C. virginica* (81%) was significantly higher than that of *C. ariakensis* (14%). At medium and high salinity sites, all *C. virginica* died before the end of the study whereas final mean cumulative mortality in *C. ariakensis* was 13 to 16%. After 1 year of deployment, mean shell height of *C. virginica* at low, moderate, and high salinity sites was respectively 70, 80 and 73 mm. In comparison, mean shell height of *C. ariakensis* was respectively 93, 121 and 137 mm. At low salinity sites, mean growth rate of *C. virginica* was not significantly different from that of *C. ariakensis*. At medium and high salinity sites, mean growth rate of *C. virginica* was significantly lower than that of *C. ariakensis*. Prevalence and intensity of *Perkinsus marinus* infections were significantly higher in *C. virginica* than in *C. ariakensis*. During the second summer of disease exposure, prevalence in *C. virginica* was 100% at all sites whereas in *C. ariakensis* it ranged from 0 to 28%. Heavy intensity of infections were prevalent in *C. virginica* whereas infections in *C. ariakensis* were limited to light intensity. *Haplosporidium nelsoni* (MSX) was present in *C. virginica*, but absent in *C. ariakensis*. Mud worms (*Polydora* spp.) were present in both oyster species, but infestations were low and did not appear to affect condition or growth. In summary, wide salinity tolerance and low disease susceptibility were associated with high survival and growth of *C. ariakensis* in Chesapeake Bay and the Atlantic Coast of Virginia.

KEY WORDS: Chesapeake Bay, aquaculture, species introduction, *Crassostrea ariakensis*, *C. virginica*

INTRODUCTION

In contrast to extensive information available on the eastern oyster *Crassostrea virginica*, and Pacific oyster *Crassostrea gigas*, reports on the Suminoe oyster *Crassostrea ariakensis* (= *C. rivularis*), are scarce. Suminoe oysters have been reported to be naturally distributed from southern Japan along the south China coast through southeast Asia to the western coast of the Indian subcontinent, but the taxonomy is tenuous in some areas and its actual distribution not clearly known (Carriker & Gaffney 1996).

Larval settlement for *C. ariakensis* is reported to occur primarily in estuarine areas with low salinity, but juvenile and adult oysters grow within a wide range of salinity (Guo et al. 1999, Ahmed et al. 1987, Cai et al. 1992). Cultivation is important in southern China using seed oysters collected from the wild (Guo et al. 1999). On the West Coast of USA, where *C. ariakensis* was introduced with shipments of *C. gigas* and kumamoto oysters (*Crassostrea sikamea*, Anemiya 1928) from southern Japan in the 1970s (Breese and Malouf 1977), its aquaculture potential has been established (Langdon and Robinson 1996). Using field experiments to compare the growth of *C. ariakensis* and *C. gigas*, Langdon and Robinson (1996) found that both species had similar growth and meat condition at various locations along the West Coast.

To the best of our knowledge, no parasitic diseases have been reported in Suminoe oysters within its native range. However, in Zhanjiang Bay, southern China, mass mortality of *C. ariakensis* has been associated with outbreaks of toxic phytoplankton blooms (Yongjia et al. 1995). In Marennes Oleron, France, mortality in association with *Bonamia*-like parasites was observed in quarantined *C. ariakensis* animals exposed to *Bonamia ostreae* endemic waters (Cochennec et al. 1998).

Studies on the potential performance and disease susceptibility of Suminoe oysters are not available for the Atlantic Coast of USA. However, as native eastern oyster stocks collapsed throughout much of the mid-Atlantic seaboard due to over harvesting, disease, and water quality deterioration, interest in the potential use of non-native oyster species has grown. Following a Virginia program to examine the suitability of non-indigenous oyster species to the local environments (VIMS 1996), *C. gigas* was the first species to be evaluated in Chesapeake Bay and the Atlantic Coast of Virginia (Calvo et al. 1999). Over the course of that study, from May 1997 to 1998, *C. gigas* had lower disease susceptibility than *C. virginica*, but survival and growth were equal or superior in native oysters than in *C. gigas* within Chesapeake Bay. Based on its close resemblance to the native oyster and its tolerance of temperate to sub-tropical environments, *C. ariakensis* was the second candidate species selected for testing in Virginia (VIMS 1996). Considering its documented ability to grow in a wide range of salinity, we hypothesized that *C. ariakensis* would perform better relative to *C. virginica* than had *C. gigas* in Chesapeake Bay. The objectives of the present study were to compare survival, growth, disease susceptibility and infestations by shell boring polychaetes in *C. ariakensis* and *C. virginica* deployed over a range of salinity.

MATERIALS AND METHODS

Study Sites

Six sites were selected based several criteria including salinity regime, geographic location, available information on oyster growing conditions and water quality, safety, logistics, and relevance

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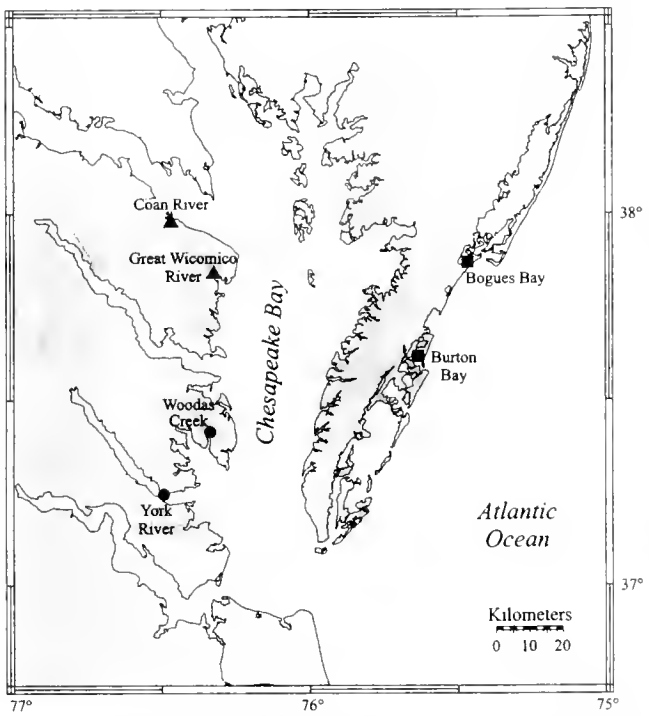

Figure 1. Location of study sites in Chesapeake Bay and the Atlantic Coast of Virginia: ▲ = Low salinity (<15‰) sites, ● = Medium salinity (15–25‰) sites, ■ = High salinity (>25‰) sites.

for the oyster industry. Sites were established at duplicate locations within low salinity (<15‰), medium salinity (15–25‰), and high salinity (>25‰) areas (Fig 1). Low and medium salinity sites were established near the margins of rivers (Coan, Great Wicomico, and York) or in shallow creeks surrounded by marshes (Woodas Creek, a tributary of the East River). High salinity sites were located in well-flushed narrow channels surrounded by marshes and mudflats in the coastal lagoon system of the Atlantic Coast of Virginia.

Temperature and salinity were measured during monthly site visits with a stem thermometer and a refractometer. To further characterize environmental variables, hourly temperature, salinity, and turbidity were measured with Hydrolab-Minisonde® dataloggers deployed at the same sites for weekly to monthly intervals.

Oysters

Individually certified triploid *C. ariakensis* were produced and maintained in quarantine first at the Haskin Shellfish Research Laboratory, Rutgers University (HSRL) and then at the Virginia Institute of Marine Science's (VIMS) Aquaculture Genetics and Breeding Technology Center. *Crassostrea ariakensis* brood stock, originating from an established line maintained in quarantine at HSRL and derived from sources on the West Coast of USA, was spawned in July 1996. Triploidy was induced by treatment of fertilized eggs with cytochalasin-B using the methods described by Downing and Allen (1987) and Allen et al. (1989). Juvenile *C. ariakensis* were transferred to flow-through York River water with quarantined effluents at VIMS, where oysters were maintained until they were individually examined for triploidy, as described later, before field deployment in early 1998. *Crassostrea virginica* brood stock, collected from Mobjack Bay, VA was spawned by a local commercial hatchery (Middle Peninsula Aquaculture) in July 1996. Prior to deployment, diploid *C. virginica* juveniles were maintained by Mobjack Bay Seafood in the Ware River, VA.

Experimental Design

Between May 29 and June 2, 1998, adult oysters were dispensed into replicate 9.5-mm mesh bags and placed within individual floating trays at the study sites. There were two replicate sites within each of three salinity regimes (Fig. 1). Each floating tray contained two bags with 100 oysters and one bag containing 50 individually labeled oysters, to follow growth, as described later. Floating trays (2.3 m × 0.5 m × 0.3 m) were constructed by fitting wire mesh trays (25-mm square 16 gauge mesh) into floating frames built with 4-inch (10.16 cm) PVC pipe, following the design of Luckenbach and Taylor (1997). Floating trays and bags were cleaned of fouling organisms at least once a month during regular site visits and more often if necessary. All sites were visited monthly (± 15 days).

Mortality, Growth and Condition

All live and dead oysters within each float were counted monthly to determine survival. Monthly mortality was calculated as the number of oysters that died during each month interval, divided by the number of live oysters at the beginning of the month, corrected for oysters removed by sampling. Cumulative mortality was calculated as the sum of interval mortality (Barber and Mann 1994, Krebs 1972). Mortality data was examined for normality and homogeneity of variance using plots of means versus standard deviations and Bartlett's chi-square test (Zar 1974). A two-way ANOVA was employed to examine the effects of species and salinity on arcsin-transformed cumulative mortality. Statistical analyses were performed using Statview® and Statistica® software.

To follow growth, 50 oysters within each float were individually labeled and shell height was repeatedly measured to the nearest 0.1 mm using calipers, once monthly except January, February, and April 1999. Monthly growth rates for individual oysters were calculated as the overall shell height increment during the growing period while live oysters of both species were still available at all sites, June 1998 to May 1999, and divided by the deployment time in days standardized to 30 days. When oysters died measurements were taken from the remaining individuals without replacement. Growth rate data was examined for normality and homogeneity of variance using the same tests specified above for mortality data. The effects of species and salinity regime on arcsin-transformed mean growth rate were examined using a two-way ANOVA followed by a Newman-Keuls test.

At the end of the experiment, in September 1999, whole weight, shell weight, tissue wet and dry weights were measured on the same oysters collected for disease diagnoses. Following Lawrence and Scott (1982), Condition Index (CI) was calculated by the formula:

$$CI = \text{tissue dry weight} * 100 / (\text{whole weight} - \text{shell weight}). \quad (1)$$

Oysters were allowed to air-dry for 15 to 20 minutes before weighing and whole oyster weight was recorded to the nearest 0.01g. Oysters were then shucked, shells weighed to the nearest 0.01g, and wet tissues were gently rolled on a paper towel and weighed on pre-tarred vessels to the nearest 0.001g. Wet tissues were dried at 80°C overnight and tissue dry weight was measured the next day

to the nearest 0.001g. Condition index data was examined for normality and homogeneity of variance using the same tests specified above for mortality data. Non-parametric statistics were employed because means and standard deviations were still highly correlated ($r = 0.952$) after transformation. Mann-Whitney tests were used to examine differences in mean condition index and mean ranked body weights between species. Kruskal-Wallis tests were employed to examine differences in the above parameters among salinity regimes.

Diseases and Polydora

A baseline sample of 25 oysters was taken to assess the disease status of each species prior to deployment in May 1998. Subsequent samples of each species at each site were collected in August and September 1998, and in May, August, and September 1999. *Perkinsus marinus* was diagnosed using Ray's fluid Thioglycollate medium (RFTM) assays (Ray 1952) on combined mantle, gill, and rectal tissue. Infection intensity was rated based on Ray (1954) and Mackin (1962). For the calculation of weighted prevalence infection intensity was ranked, following Paynter and Burreson (1991), as: 0 = negative, 1 = light, 3 = moderate and 5 = heavy. Light-moderate infections were ranked as 1 and moderate-heavy infections were ranked as 3. Weighted prevalence was calculated by the formula:

$$\text{Weighted prevalence} = \Sigma I^{*} n_I / N \qquad (2)$$

Where I = infection intensity rank
n_I = number of oysters within I
N = total number of oysters examined in the sample.

Prevalence and weighted prevalence data were examined for normality and homogeneity of variance using the same tests specified above for mortality data. Two-way ANOVAs followed by Newman-Keuls tests were employed to analyze the effects of species and salinity regime on arcsin-transformed prevalence and untransformed weighted prevalence.

Haplosporidium nelsoni, the causative agent of MSX disease, was diagnosed using standard paraffin histology procedures with oysters preserved in Davidson's AFA and 6 μm tissue sections stained with Harris' hematoxylin and eosin (Burreson et al. 1988). Infection intensity was rated as light, moderate or heavy based on Burreson et al. (1988). Histology sections were also used to document the presence of other parasites and to examine development of oyster gonads. Disease diagnoses and histology were performed by the VIMS Shellfish Pathology Laboratory.

The spionid polychaetes *Polydora websteri* and *P. ligni* are commensal with bivalves, including oysters. These suspension-feeding worms do not feed on the oyster, but the mechanical irritation caused by their presence causes the oyster to lay down additional layers of conchiolin over the worm's tube in what are often termed mud-blisters. At sufficiently high levels of infestation this can severely limit the growth of oysters and reduce their condition index. Examination for mud-blisters associated with *Polydora* spp. was conducted on the same oysters collected for condition and disease diagnoses in September 1999. Worms were not identified to species, but *Polydora websteri* is the most common species affecting oysters in the northeast coast of the United States (Blake and Evans 1972, Wargo and Ford 1993). The internal surface of right valve shells was visually inspected and rated according to the presence and extent of mud-blisters. Examination was restricted to right valves as in Wargo and Ford (1993) who reported that infestations by *Polydora* spp. were equally found in right and left valves. Following the methods of Handley and Bergquist (1997), infestation was rated as: (0) no visible mud-blisters or any evidence of boring by *Polydora* spp.; (1) mud-blisters affecting less than 25% of the valve; (2) 25–50% of the valve affected; (3) 50–75% of the valve affected; or (4) more than 75% of the valve affected. Weighted prevalence was calculated similarly to equation (2) using the five categories above. Prevalence and weighted prevalence data was examined for normality and homogeneity of variance using the same tests specified above for mortality data. Non-parametric tests were employed because zero variances precluded computation of Bartlett's test for determining homogeneity of variance. Mann-Whitney tests were used to examine differences in mean prevalence and mean weighted prevalence between species. Kruskal-Wallis tests were employed to examine differences in the above parameters among salinity regimes.

Reproductive Status and Ploidy

A baseline sample of *C. ariakensis* was collected to ascertain the percentage of triploid individuals in the lot of animals exposed to Cytochalasin-B as described above. Prior to field deployment, all *C. ariakensis* animals were individually certified as triploids following flow-cytometric methods (Allen 1983). Briefly, experimental animals were notched on the dorsal side of the right valve using a Dremel® rotary tool equipped with a fiberglass-cutting wheel. A 1- ml syringe fitted with a 23-gauge needle was inserted into the adductor muscle and 0.05 ml of hemolymph was removed. A 10 μg/ml DAPI-10% DMSO staining solution was added to the hemolymph and the sample was vortexed, aspirated, and filtered through a 23μm Nitex® screen. DNA content of prepared samples was determined on a PARTEC® Cell Cycle Analyzer via ultraviolet light excitation. Histograms of relative DNA content were used to identify diploid cells with modal DNA values 1.5 times lower than that of triploid cells. Individuals with triploid and diploid cells were categorized as mosaics. A 2mm × 2mm piece of gill tissue, as well as a cross-section of gonad tissue were also sampled from mosaic individuals and examined for DNA content as above. The remaining gonad from mosaic individuals was processed by histology.

Over the course of the experiment, samples of *C. ariakensis* (n = 16–35) were collected from each site in July and August 1998 and in May, June, and July 1999. Ploidy assays were conducted at HSRL and the VIMS Aquaculture Genetics and Breeding Technology Center. A two-way ANOVA was employed to examine the effects of salinity and time on mean arcsin-transformed percentage of mosaics. Significant effects were further examined using a Newman-Keuls test.

RESULTS

Environmental Parameters

Means of monthly salinity measures at the two low salinity sites were below 10‰ only during June and July 1998. Drought conditions prevailed throughout much of the study and salinities above 15‰ were recorded at the low salinity sites from November 1998 to March 1999. Medium salinity sites experienced relatively low salinity (<15‰) during June 1998, but were between 15 to 25‰ on all other sampling dates (Fig. 2). Salinity fluctuations in

high salinity sites were within the expected range (25–35‰). Temperature followed similar seasonal trends at all sites with a maximum of 28° to 32°C in July and a minimum of 0° to 5°C in March. High salinity sites experienced overall cooler temperature with monthly means 2° to 4°C lower than medium or low salinity sites (Fig. 2). Turbidity was low (<70 Nephelometric turbidity units at all sites and times tested). Dissolved oxygen was relatively low (60% air-saturation) at Woodas Creek in July 1999 compared to all other sites and times measured (68–87% air-saturation).

Mortality

As the experiment progressed, *C. virginica* percent cumulative mortality rapidly increased whereas *C. ariakensis* mortality remained low. The highest increase in mean cumulative mortality, from 5% to 78%, was observed in *C. virginica* at medium salinity between July and October 1998 (Fig. 3). At the end of the experiment, mean cumulative mortality in *C. virginica* (81%–100%) was significantly higher ($p < 0.0005$) than that in *C. ariakensis* (13%–16%). Salinity had a significant ($p = 0.006$) effect on final cumulative mortality. The interactive effect of species and salinity was also significant ($p = 0.011$) and may be attributed to the increase in mortality between low and higher salinities observed for *C. virginica* whereas low mortality was similarly observed for *C. ariakensis* at all salinities (Fig. 4).

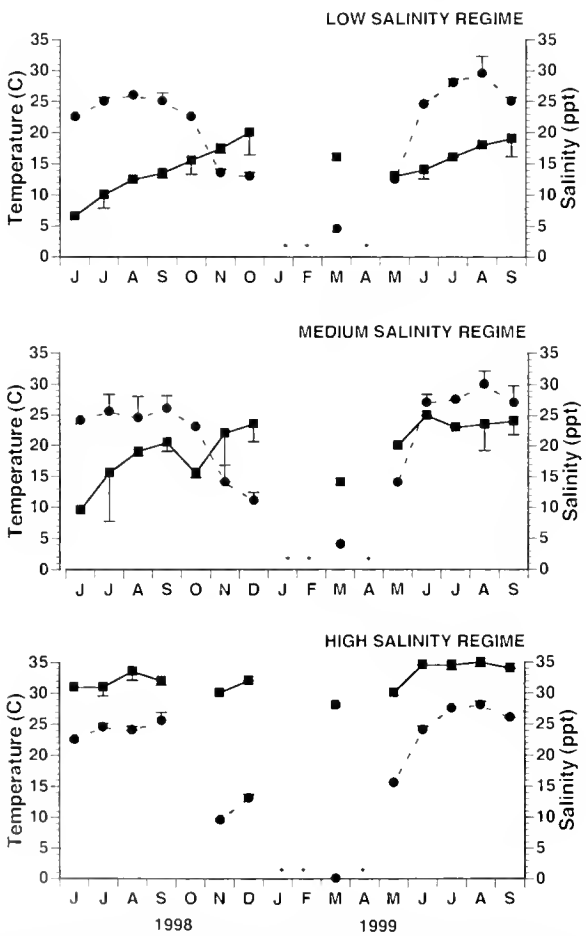

Figure 2. Means of monthly temperature and salinity measurements by salinity regime (N = 2 sites, ± SD), from June 1998 to September 1999. ● = Temperature using stem thermometer. ■ = Salinity using refractometer.

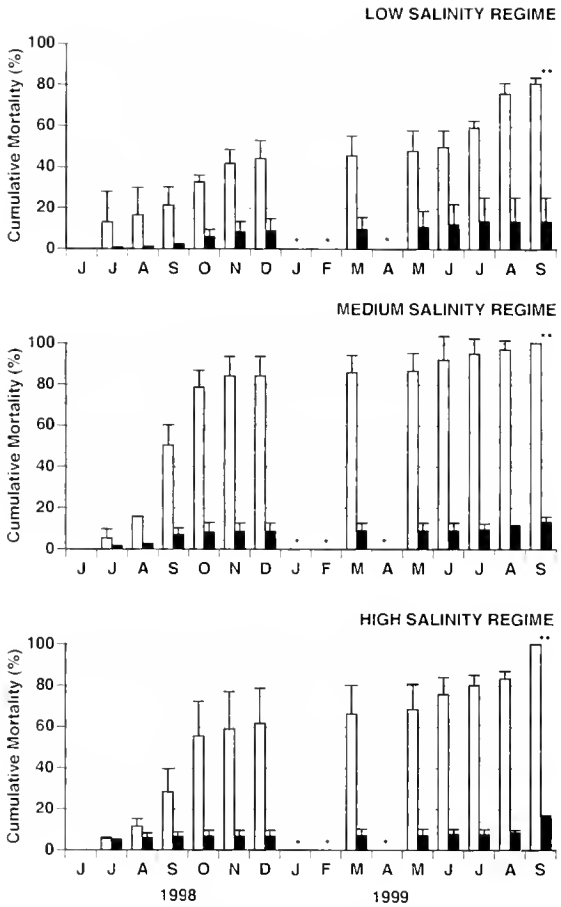

Figure 3. Mean cumulative mortality by salinity regime (N = 2 sites, + SD) from May 1998 to September 1999. Open bars = *C. virginica*. Solid bars = *C. ariakensis*. ** = Significant at $\alpha = 0.01$. NS = Not sampled.

Growth

Growth varied with species and salinity regime (Fig. 5 and Table 1). At the start of the experiment mean shell height was 60 mm in *C. virginica* and 64 mm in *C. ariakensis*. After 1 year of deployment, in May 1999, mean shell height of *C. virginica* at low, medium, and high salinity sites was respectively 70, 80 and 73 mm. In comparison, mean shell height of *C. ariakensis* at low,

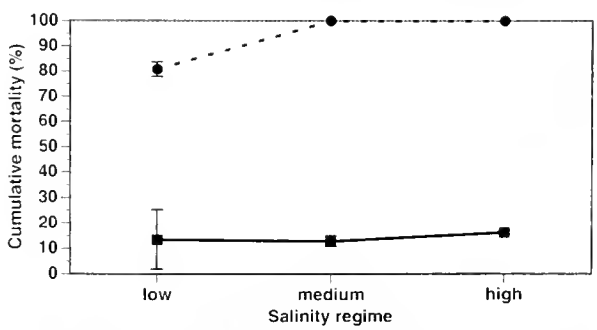

Figure 4. Interaction between oyster species and salinity on final cumulative mortality. Means of 2 sites (± SD). ● = *C. virginica*. ■ = *C. ariakensis*.

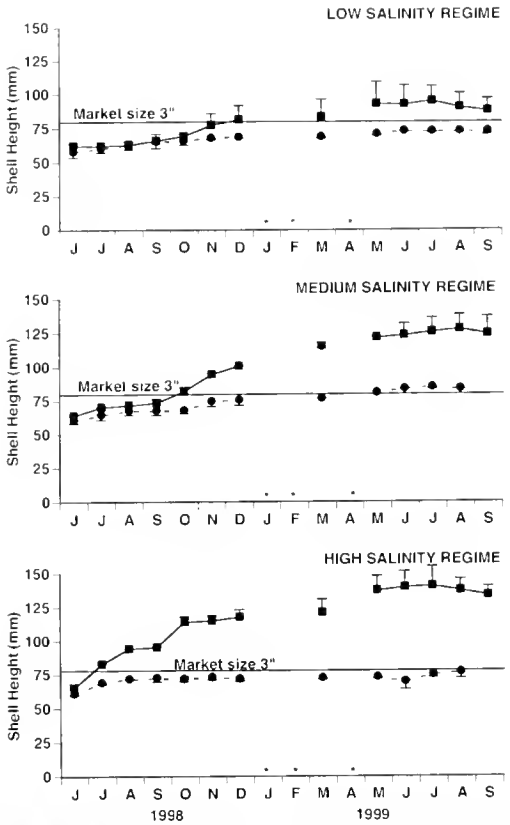

Figure 5. Mean shell height by salinity regime (N = 2 sites, ± SD) of 50 individual oysters repeatedly measured from May 1998 to September 1999. ● = *C. virginica*. ■ = *C. ariakensis*.

moderate, and high salinity sites, was respectively 93, 121 and 137 mm. Oysters continued to grow until July 1999 when mortality of *C. virginica* approached 100% in all medium and high salinity sites. At that time mean shell height *of C. ariakensis* at low, medium and high salinity sites was respectively, 96, 125 and 140 mm. No growth was observed for either species during July to September 1999. Most of the growth occurred during fall 1998 and spring 1999.

Species and salinity regime had a significant effect on mean growth rate (Table 1A). Similar growth rates were observed for *C. virginica* at all salinities in contrast to increasing growth rates with increasing salinity observed for *C. ariakensis* (Fig 6). At low salinity sites, mean growth rate of *C. virginica* (1.1 mm mo^{-1}) was not significantly different than that of *C. ariakensis* (2.6 mm mo^{-1}). At medium salinity sites, mean growth rate of *C. virginica* (1.7 mm mo^{-1}) was significantly lower than that of *C. ariakensis* (4.9 mm mo^{-1}). At high salinity sites, mean growth rate of *C. virginica* (1.0 mm mo^{-1}) was also significantly lower than that of *C. ariakensis* (6.2 mm mo^{-1}). For *C. virginica*, growth rate did not differ significantly among salinity regimes. For *C. ariakensis*, however, growth rate at low salinity was significantly lower than that at medium and high salinity regimes (Table 1B).

Disease

At the beginning of the study, there was no *P. marinus* and a 4% prevalence of *H. nelsoni* in *C. virginica* and 12% prevalence of *P. marinus* and no *H. nelsoni* in *C. ariakensis* (Fig. 7). In October 1998, prevalence and weighted prevalence of *P. marinus* were significantly higher ($p < 0.0005$) in *C. virginica* than in *C. ariakensis*. In September 1999, when no live *C. virginica* remained at the medium salinity York River site, prevalence in *C. virginica* at all other sites was 100% whereas prevalence in *C. ariakensis* ranged from 0 to 75% and did not differ ($p > 0.250$) among salinity regimes (Fig. 7). Heavy infections were prevalent in *C. virginica* whereas only light infections were observed in *C. ariakensis* (Table 2).

Maximum prevalence of *H. nelsoni* (25%) was observed in *C. virginica* at the York River site in May 1999. *H. nelsoni* was also present in *C. virginica* at the low salinity Great Wicomico River site in September 1998 (4%), and at high salinity sites in October 1998 (4–8%) and May 1999 (4%). Intensity of *H. nelsoni* infections was light except for heavy infections found in oysters sampled from medium (1/132) and high salinity sites (1/157). No *H. nelsoni* was found in *C. ariakensis*. Other parasites observed in histological sections of *C. virginica* were the protozoan *Haplospo-*

TABLE 1.

Effects of species and salinity regime on mean growth rate.

A. Two-way ANOVA

Effect	df	MS	F	p
Species	1	32.293	61.382	<0.0005
Salinity	2	3.441	6.536	0.052
Species*Salinity	2	3.225	6.124	0.076
Error	6	0.526		

B. Multiple comparison (Newman-Keuls test)

Comparison		
Within	**Between**	**p**
Low salinity	*C. virginica* and *C. ariakensis*	0.116
Medium salinity	*C. virginica* and *C. ariakensis*	0.018
High salinity	*C. virginica* and *C. ariakensis*	0.005
C. virginica	Low salinity vs. medium salinity	0.268
C. virginica	Low salinity vs. high salinity	0.931
C. virginica	Medium salinity vs. high salinity	0.440
C. ariakensis	Low salinity vs. medium salinity	0.034
C. ariakensis	Low salinity vs. high salinity	0.018
C. ariakensis	Medium salinity vs. high salinity	0.280

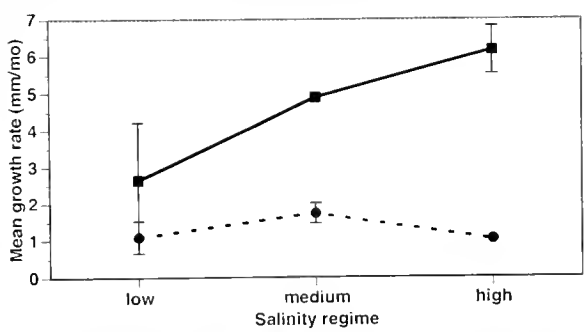

Figure 6. Interaction between oyster species and salinity on mean growth rate. Means of 2 sites (± SD). ● = *C. virginica*. ■ = *C. ariakensis*.

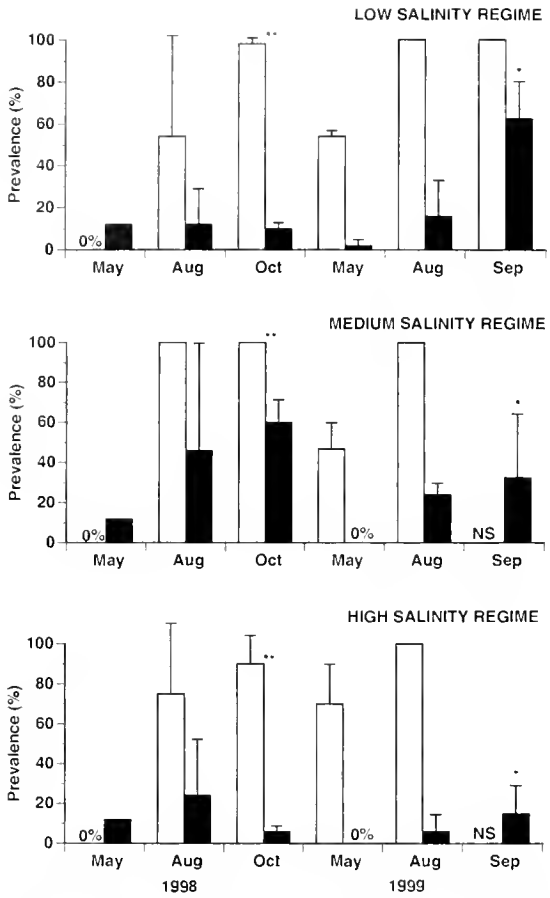

Figure 7. Mean prevalence of *P. marinus* by salinity regime (N = 2 sites, + SD), in samples of 25 oysters, from May 1998 to September 1999. Open bars = *C. virginica*. Solid bars = *C. ariakensis*. ** = Significant at $\alpha = 0.01$. * = Not significant at $\alpha = 0.05$. NS = Not sampled because no *C. virginica* remained.

ridium costale (SSO), present at high salinity sites, the trematode *Bucephalus* sp. and a chlamydia-like organism. None of these or other parasites were observed in *C. ariakensis*.

Condition

At low salinity sites, mean condition index in *C. virginica* and *C. ariakensis* were, 3.6 and 6.6, respectively, and means were not significantly different ($p = 0.121$). Similarly, there were no significant differences ($p = 0.121$) in mean body weights between species. At medium and high salinity, comparisons between species were not possible because no live *C. virginica* remained at those sites at the end of the experiment. For *C. ariakensis*, mean condition index at low, medium and high salinity, respectively, were 6.6, 5.3 and 9.7 and means were not significantly different ($p = 0.276$). Similarly, there were no significant differences ($p > 0.102$) between mean body weights among salinity regimes. The percentage of shell weight relative to whole oyster weight in *C. virginica* (62%) was similar to that in *C. ariakensis* at low, medium, or high salinity, respectively, 59, 61 and 65%.

Polydora

At low salinity sites, mean prevalence of *Polydora* spp. was 100% in both oyster species, and there was no significant difference in mean weighted prevalence between oyster species ($p = 0.121$). At medium and high salinity, comparisons between species were not possible because at the end of the experiment there were no live *C. virginica* at those sites. For *C. ariakensis*, there was a trend of decreasing prevalence with increasing salinity. Mean prevalence in *C. ariakensis* at low, medium and high salinity sites were, respectively, 100, 62 and 12%. However, ranked mean prevalence and weighted prevalence were not significantly different ($p = 0.156$) among salinity regimes.

Ploidy

The baseline sample revealed that prior to deployment 94% of the *C. ariakensis* in the lot were triploids. Individual certification assured that triploids were exclusively deployed in the field. During the course of the study, there were 62 individuals in which combinations of diploid and triploid cells (mosaics) were detected out of 1164 oysters examined (5.3%). The proportion of mosaics ranged from 0.0 to 13.8% depending on time and site (Table 3). The effect of time on mean percentage of mosaics was significant ($p = 0.002$). Mean percentage of mosaics increased significantly between the first sampling time and each subsequent sampling time ($p < 0.007$). After the initial sampling time, the percentage of mosaics did not differ significantly among the remaining sampling times ($p > 0.498$). Salinity regime had no effect on mean percentage of mosaics ($p = 0.128$). Examination of 39 mosaic individuals revealed that 10 were females, 23 were males, one was hermaphroditic, and five were undifferentiated.

DISCUSSION

Drought conditions and below normal Chesapeake Bay stream flow starting in fall 1998 resulted in increased salinity and epizootics of both *H. nelsoni* and *P. marinus* during 1999 (Ragone Calvo & Burreson 1999). High disease pressure prevailing in the region was associated with severe infections and high mortality in *C. virginica*, though not in *C. ariakensis*. After the first summer of disease exposure, more than 50% of *C. virginica* had died and prevalence of *P. marinus* at medium and high salinity sites was 100%. A year later when all *C. virginica* at medium and high salinity sites were dead, cumulative mortality at low salinity sites was 81% and prevalence of *P. marinus* was 100% with severe infections present. Maximum prevalence of *H. nelsoni* in *C. virginica* was 25% whereas no *H. nelsoni* was detected in *C. ariakensis*. Presence of *H. nelsoni* and intensification of *P. marinus* infections in *C. virginica* at the low salinity Great Wicomico site was undoubtedly favored by drought conditions resulting in salinity greater than 15% starting in fall 1998 and continuing into spring and summer 1999. Persistence of salinity greater than 15% during summer and fall is conducive to development of lethal *P. marinus* infections (Burreson and Ragone Calvo 1996). In comparison, maximum *P. marinus* prevalence in *C. ariakensis* reached 84%, but infections never exceeded light intensity and mortality remained low (13–16%). With the caveat that this study spanned only 15 months, *C. ariakensis* appears highly tolerant of the dominant parasitic diseases affecting Chesapeake Bay oysters.

A limitation of this study was that conditions were not identical for both species before the beginning of the experiment. Since *C. ariakensis* was quarantined for their first two years in land-based systems with limited inflow of raw York River water, long-term exposure to disease agents may have been reduced in relation to

TABLE 2.

Prevalence and intensity of *P. marinus* in *C. virginica* and in *C. ariakensis* by salinity regime, site and date during 1998 (A) and 1999 (B).

A.

			C. virginica				*C. ariakensis*			
Salinity	**Site**	**Date**	**Prevalence†**	**L***	**M***	**H***	**Prevalence†**	**L***	**M***	**H***
L	CNRV	8/12/98	20% (5/25)	3	2	0	0% (0/25)	0	0	0
		9/30/98	96% (24/25)	18	2	4	12% (3/25)	3	0	0
	GWRV	8/4/98	88% (22/25)	21	0	1	24% (6/25)	6	0	0
		9/30/98	100% (25/25)	12	4	9	28% (7/25)	7	0	0
M	WOCK	8/3/98	100% (25/25)	7	5	13	84% (21/25)	21	0	0
		9/30/98	100% (24/24)	7	7	10	68% (17/25)	17	0	0
	YKRV	8/3/98	100% (25/25)	16	3	6	8% (2/25)	2	0	0
		9/29/98	100% (25/25)	7	11	7	52% (13/25)	13	0	0
H	BUBY	8/6/98	100% (25/25)	20	1	4	44% (11/25)	11	0	0
		10/7/98	80% (20/25)	13	6	1	8% (2/25)	2	0	0
	BOBY	8/6/98	50% (25/50)	19	4	2	4% (1/25)	1	0	0
		10/13/98	100% (25/25)	13	7	5	4% (1/25)	1	0	0

B.

			C. virginica				*C. ariakensis*			
Salinity	**Site**	**Date**	**Prevalence†**	**L***	**M***	**H***	**Prevalence†**	**L***	**M***	**H***
L	CNRV	5/3/99	52% (13/25)	12	0	1	4% (1/25)	1	0	0
		8/2/99	100% (25/25)	10	12	3	4% (1/25)	1	0	0
		9/21/99	100% (14/14)	4	4	6	50% (6/12)	6	0	0
	GWRV	5/3/99	56% (14/25)	11	2	1	0% (0/25)	0	0	0
		8/2/99	100% (24/24)	9	5	10	28% (7/25)	7	0	0
		9/21/99	100% (6/6)	1	1	4	75% (15/20)	15	0	0
M	WOCK	5/5/99	56% (14/25)	11	1	2	0% (0/25)	0	0	0
		8/2/99	100% (3/3)	0	1	2	28% (7/25)	7	0	0
		9/22/99	NS	–	–	–	55% (11/20)	11	0	0
	YKRV	5/4/99	37% (3/8)	3	0	0	0% (0/25)	0	0	0
		8/3/99	NS	–	–	–	19% (4/21)	4	0	0
		9/21/99	NS	–	–	–	10% (2/20)	2	0	0
H	BUBY	5/6/99	84% (21/25)	19	0	2	0% (0/25)	0	0	0
		8/5/99	100% (13/13)	12	0	1	12% (3/25)	3	0	0
		9/2/99	NS	–	–	–	25% (5/20)	5	0	0
	BOBY	5/6/99	56% (14/25)	13	0	1	0% (0/25)	0	0	0
		8/4/99	100% (25/25)	19	4	2	0% (0/25)	0	0	0
		9/21/99	NS	–	–	–	0% (0/25)	0	0	0

Salinity codes: L = low (<15‰), M = medium (15–25‰), H = high (>25‰). Site codes: CNRV = Coan River, GWRV = Great Wicomico River, WOCK = Woodas Creek, YKRV = York River, BUBY = Burton Bay, BOBY = Bogues Bay. † = In parenthesis number of oysters infected/number of oysters examined. * = Number of oysters with, respectively, light, moderate, and heavy infections. NS = No *C. virginica* remaining for sampling.

that of *C. virginica* which was initially maintained in the water at a nearby location. Absence of *P. marinus* in the baseline sample of *C. virginica* collected in May 1998 does not preclude the possibility of sub-clinical infections. Given the low sensitivity of the standard diagnostic assay for detecting *P. marinus* infections during spring (Bushek et al. 1994, Burreson and Ragone Calvo 1996), it is likely that acquisition of infections occurred the prior summer but that overwintering infections were not detected the following spring.

Suminoe oysters tested in this study had comparable survival at all salinity regimes and similar growth rate at medium and high salinity regimes, in agreement with the wide salinity tolerance described for *C. ariakensis* in its native range (Guo et al. 1999). By the end of the experiment, when oysters were three years old, mean shell height of *C. ariakensis* at low, medium, and high salinity regimes was respectively 96, 125 and 140 mm. By comparison, in Zhanjiang Bay (annual salinity range = 7–30‰) average shell height of three-year-old Suminoe oysters is 100 mm (Cai et al. 1992).

In contrast to *C. gigas* in previous studies (e.g., Handley & Berquist 1997, Calvo et al. 1999), *C ariakensis* was not found to be adversely affected by *Polydora* spp. in this study. Mud worms were present in both *C. ariakensis* and *C. virginica*, but infestations were not severe and did not appear to affect growth or survival of either species. However, since the conditions that result in severe infestations are not clearly understood, we cannot dismiss the possibility that *C. ariakensis* might be susceptible to such infestations.

Results of the present investigation suggest that *C. ariakensis* is more adapted to Chesapeake Bay conditions than *C. gigas*. In a

TABLE 3.

Percentage of genetic mosaics by salinity regime, site and time.

		1998		1999					
Salinity	**Site**	**July**	**August**	**May**	**June**	**July**	**August**	**Total by site**	**Total by salinity**
L	CNRV	0.0%	2.8%	8.6%	8.6%	9.7%	8.0%	6.1%	5.0%
		(0/35)	(1/35)	(3/35)	(3/35)	(3/31)	(2/25)	(12/196)	(20/395)
	GWRV	2.8%	2.8%	2.8%	5.7%	5.9%	4.0%	4.0%	
		(1/35)	(1/35)	(1/35)	(2/35)	(2/34)	(1/25)	(8/199)	
M	EARV	0.0%	2.8%	11.4%	8.6%	13.8%	12.0%	7.7%	7.2%
		(0/35)	(1/35)	(4/35)	(3/35)	(4/29)	(3/25)	(15/194)	(28/391)
	YKRV	0.0%	11.4%	11.4%	8.6%	2.9%	4.3%	6.6%	
		(0/35)	(4/35)	(4/35)	(3/35)	(1/34)	(1/23)	(13/197)	
H	BUBY	0.0%	8.8%	2.8%	5.7%	0.0%	8.0%	4.1%	3.7%
		(0/35)	(3/34)	(1/35)	(2/35)	(0/33)	(2/25)	(8/197)	(14/379)
	BOBY	0.0%	0.0%	2.8%	5.7%	6.2%	8.0%	3.3%	
		(0/35)	(0/35)	(1/35)	(2/35)	(1/16)	(2/25)	(6/181)	
Total by month		0.5%	4.8%	6.7%	7.1%	6.2%	7.4%		
		(1/210)	(10/209)	(14/210)	(15/210)	(11/177)	(11/148)	Overall total = 5.3% (62/1164)	

Salinity codes: L = low (<15‰), M = medium (15–25‰), H = high (>25‰). Site codes: CNRV = Coan River, GWRV = Great Wicomico River, EARV = East River, YKRV = York River, BUBY = Burton Bay, BOBY = Bogues Bay. In parenthesis number of mosaics divided by number of oysters examined.

comparative study with *C. virginica* and *C. gigas* (Calvo et al. 1999), *C. gigas* exhibited high cumulative mortality (63%) at low salinity sites and growth rate was not greater than that of *C. virginica* within Chesapeake Bay. In contrast, *C. ariakensis* tested in this study had less than 16% cumulative mortality and greater growth rate than *C. virginica* within medium salinity sites in Chesapeake Bay. In grow-out trials with *C. gigas* and *C. ariakensis* using various culture methods at high salinity locations on the West Coast of USA, growth rate was the same for both oyster species. For example, *C. gigas* and *C. ariakensis* juveniles (mean shell height = 10 mm) planted directly on the bottom in Puget Sound, Washington, or suspended from floating rafts, in Yaquina Bay, Oregon, increased to 90–100 mm after 1 year of deployment during 1990 to 1991 (Langdon & Robinson 1996). A direct comparison between *C. gigas* and *C. ariakensis* is not available for the East Coast of USA. However, in this study and in Calvo et al. (1999), both species experienced significantly higher growth rate than the corresponding *C. virginica* control oysters at high salinity sites on the Atlantic Coast of Virginia.

The choice of oyster strain must be considered in the interpretation of results. *C. virginica* offspring from wild Mobjack Bay brood stock were employed in this study because they are a standard stock for aquaculture and because they were the only stock available with similar age, size and disease status *as C. ariakensis*. It is likely that a strain of *C. virginica* selected for disease resistance would more favorably compare to *C. ariakensis*. For example, DEBY, a strain of *C. virginica* that has been selectively bred by VIMS against *P. marinus* and *H. nelsoni* for four generations (Ragone Calvo et al. 1997), exhibited similar survival to that of *C. ariakensis* and higher survival, higher growth rate and lower disease susceptibility than that of unselected *C. virginica* from Mobjack Bay deployed at a site in the Great Wicomico River from June 1998 to May 1999 (G. Calvo unpublished data). Similarly, CROSBreed strains (Debrosse and Allen 1996) have been selected for dual resistance to both pathogens in the Mid-Atlantic. Use of disease resistant *C. virginica* strains could provide a more relevant comparison of native and non-native oyster performance for aquaculture.

To some extent, the fact that *C. ariakensis* were triploids may have allowed them to grow faster than diploid *C. virginica*. In general, reduced gametogenesis in triploids corresponds with improved somatic growth (Barber & Mann 1991). However, based on studies with *C. virginica and C. gigas*, ploidy effects alone are unlikely to account for reduced disease susceptibility and increased survival of *C. ariakensis*. Barber and Mann (1991) found that cohorts of diploid and triploid *C. virginica* had the same prevalence (96%) and similar intensity of *P. marinus* after 17 months of deployment at the York River, VA. Meyers et al. (1991) reported equal cumulative mortality for diploid and triploid *C. virginica* (100%) and lower cumulative mortality for diploid (25%) than triploid *C. gigas* (34%), after one year of challenge with *P. marinus*.

In summary, during the course of the study *C. ariakensis* performed better than *C. virginica* in Chesapeake Bay and the Atlantic Coast of Virginia. Wide salinity tolerance combined with low disease susceptibility was associated with high survival and high growth rate in *C. ariakensis*. In contrast, high disease susceptibility was associated with low survival and low growth rate in *C. virginica*.

As previously discussed for *C. gigas* (Calvo et al. 1999), a decision on whether *C. ariakensis* is, or is not, an appropriate species for use in these environments must include other factors beyond the scope of these field investigations. For example, international organizations have recommended that competent local authorities consider the ecological consequences of species introductions by evaluating the following: (1) the possibility that non-indigenous species may carry pests and pathogens into the new environment; (2) the potential relationship of the exotic species with native species; and (3) the potential range for establishment of the exotic species in the new environment. A cautious introduction of triploid *C. ariakensis* for aquaculture purposes following International Council for the Exploration of the Seas guidelines, as it is

currently being considered in Virginia, would minimize risks associated with factors enumerated above. Use of individually certified triploids in a closely monitored research setting allowed us to conduct the present study with minimum risks of unintentionally introducing reproductively capable *C. ariakensis* into the waters of Virginia. At the present time, however, there is no precedent for using triploids to control oyster populations at a commercial scale. In practice, implementation of such a strategy would require significant efforts directed at monitoring the reproductive status of deployed stocks over time, and at designing and enforcing strict quarantine regulations to avoid undesired release of reproductively capable stocks.

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DEVELOPMENT AND VERIFICATION OF A MODEL FOR THE POPULATION DYNAMICS OF THE PROTISTAN PARASITE, *PERKINSUS MARINUS*, WITHIN ITS HOST, THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*, IN CHESAPEAKE BAY

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ABSTRACT A simulation model was developed to investigate the population dynamics of the protistan parasite, *Perkinsus marinus*, within its host, the eastern oyster, *Crassostrea virginica*. The main objective was to evaluate the relationship between *P. marinus* population dynamics and environmental conditions in order to predict the onset and termination of *P. marinus* epizootics in Chesapeake Bay oyster populations. Information derived from laboratory experiments and from direct field observations of *P. marinus* dynamics in the James River for the years 1990 to 1993 was utilized for model development. The individual-based model, which is driven by temperature and salinity, tracks the average within-host parasite density at a daily time step. The model was verified against monthly field observations of parasite abundance for the years 1994 to 1999 at three oyster bars located along a 0–20-ppt salinity gradient in the James River, Virginia. Simulated populations exhibited a distinct seasonal periodicity with annual density maximums and minimums occurring in October and May, respectively. Parasite abundance decreased in an upriver direction with decreasing salinity along the salinity gradient. Predicted parasite densities significantly correlated with actual observed densities at all three locations; however, the strength of the association decreased from bar to bar in an upriver direction. Predicted parasite abundance exhibited a dynamic steady state for all three oyster bars during the 6-year time series. Simulations run without the input of a midsummer transmission event resulted in a destabilization and extinction of the parasite from the oyster population located farthest upriver, but the parasite remained enzootic during the six year simulation at the two lower river stations. This suggests that a single transmission event may be sufficient for *P. marinus* to become enzootic in specific year classes of oyster populations located in moderate to high salinity areas, while periodic transmission events are required for the parasite to persist in low salinity areas. Simulation results suggest that fairly accurate quantitative predictions of *P. marinus* abundance can be made using *in situ* temperature and salinity data and a relatively simple model.

KEY WORDS: *Perkinsus*, model, disease, parasite, oyster, temperature, salinity

INTRODUCTION

Perkinsus marinus is a protistan parasite of the eastern oyster, *Crassostrea virginica*, that has significantly impacted oyster populations along the East Coast of the U.S. and in the Gulf of Mexico. Following its initial discovery in the Gulf of Mexico in 1947 (Mackin et al. 1950) *P. marinus* was soon found in the Chesapeake Bay (Andrews 1954). Prior to the mid 1980s the distribution of *P. marinus* within Chesapeake Bay was primarily restricted to moderate to high salinity areas located in the Bay and at the mouths of the major tributaries (Andrews 1988). However, the parasite's abundance dramatically increased in the late 1980s, and within a 4-year period it spread to nearly all oyster populations within the lower Bay (Burreson and Ragone Calvo 1996). Anomalous climatic conditions, consecutive drought years coincident with warm winters, are believed to be responsible for this extensive range expansion. While the historical restriction of *P. marinus* to high salinity areas suggests that over the long term the parasite cannot tolerate low salinity, the parasite remains enzootic in most low salinity areas despite the return to normal and even below normal salinities during the last decade. The persistence of the parasite in upper tributary oyster populations is particularly problematic, as these areas are the principle areas for seed production that historically has been the foundation of a significant private fishery. Critical to development of disease mitigation strategies and predictive capabilities is an understanding of how environmental factors effect *P. marinus* disease dynamics.

Many studies have focused on the importance of environmental factors in the maintenance of host-parasite equilibriums (Rohde 1982, Grenfell and Dobson 1995). The simplest conceptual model of disease dynamics is frequently drawn as three overlapping circles representing numerous host, parasite, and environmental factors. The overlapping area represents the interaction of the factors that ultimately leads to the domain of a particular disease. In a stabilized system, interactions between host, parasite, and environmental factors result in equilibrium between host and parasite populations (Anderson and May 1978). Alterations in conditions affecting any of the three components of the system can potentially result in destabilization resulting in parasite extinction or the initiation of an epizootic. Gaining an understanding of how environmental factors effect host-parasite relationships is often a challenge, but such an understanding is essential for predicting the onset and abatement of disease epizootics.

In the case of *P. marinus*, temperature and salinity have been identified as the most important environmental factors affecting its interactions with its host *C. virginica* (Andrews 1988, Burreson and Ragone Calvo 1996). Temperature appears to be the most important factor affecting the large-scale geographic distribution of the parasite (Quick and Mackin 1971, Burreson and Ragone Calvo 1996, Ford 1996) and is primarily responsible for the seasonal periodicity of *P. marinus* observed within a particular geographic area. In the Chesapeake Bay *P. marinus* exhibits a distinct seasonal periodicity in which prevalence and intensity increases during the summer months to annual maximums in September and October and then decreases during the late fall, winter, and early spring to annual minimums in April and May. The relative abundance of the parasite within an estuary at any particular time is largely influenced by salinity. Generally, *P. marinus* infections

remain light in intensity and no oyster mortality results if salinity is consistently less than 9 ppt, while high intensity infections and associated oyster mortality often occur in areas where salinities are greater than 12–15 ppt (Ragone and Burreson 1993, Burreson and Ragone Calvo 1996).

Although the independent effects of temperature and salinity on *P. marinus* have been extensively studied, fewer studies have addressed the importance of the interaction of temperature and salinity, or the interaction of temperature and salinity with other factors, in triggering and maintaining *P. marinus* epizootics. Based on simulations using an energetics-based model of *P. marinus* and *C. virginica* population dynamics in the Gulf of Mexico, Powell et al. (1996) concluded that changes in temperature and salinity alone do not trigger epizootics. Their simulations indicated that some other factor, such as reduced food supply or reduced recruitment rates, must occur prior to or coincidentally with high salinity or high temperature conditions for an epizootic to be triggered.

In the present study, a mathematical model was developed to synthesize the results of various field and laboratory studies that have been conducted in our laboratory during the last 10 years with a focus of gaining a better understanding of the interactive effect of temperature and salinity on *P. marinus* disease dynamics. We present the results of initial efforts to integrate, via an individual-based simulation model, temperature and salinity control of within-host population dynamics of *P. marinus* in the lower Chesapeake Bay.

METHODS

General Model Description

The *P. marinus* model was developed using the program STELLA II (Version 3.04, High Performance Systems Inc., Hanover, NH) on a Power Macintosh computer. The model is an individual-based model that simulates the transmission, growth and death of parasite cells within its host, the eastern oyster. Within-host parasite abundance, the only state variable, is tracked through time using a daily time step. Within-host parasite abundance represents the average abundance for a particular population of one year and older oysters. The model has four main components: parasite growth, parasite transmission, parasite mortality, and parasite loss via host death (Fig. 1). Both parasite division and parasite mortality are forced by temperature and salinity conditions. The basic model equation is (Eq. 1):

$$dP/dt = P(P_G + P_T - P_M - P_L) \quad (1)$$

where P = parasite density (cells (g wet wt)$^{-1}$), P_G = parasite cell growth, P_T = parasite transmission, P_M = parasite mortality, and P_L = parasite loss via host death (Table 1).

Perkinsus marinus Growth

Estimates of *P. marinus* growth rates or doubling times and the effects of temperature, salinity, and cell density on *P. marinus* growth are limited. Doubling time estimates derived from *in vivo* studies conducted with oysters from the Gulf of Mexico range from 7–60 hours (Saunders et al. 1993). Ford et al. (1999) found *in vivo* parasite doubling times in Delaware Bay oysters held under experimental conditions to range from 3–23 days. *In vitro* doubling times of cultured *P. marinus* have been reported to range from 13–24 hours (Dungan and Hamilton 1995, Gauthier and Vasta 1995, La Peyre 1996). Reported doubling times from both *in vivo* and *in vitro* studies are based on a variety of techniques and primarily represent optimal conditions, i.e., temperatures >25°C, high salinity (>17 ppt), and/or low initial cell densities. Alteration of any of these factors will result in changes in *P. marinus* growth rates. Saunders et al. (1993) and Ford et al. (1999) found that cell densities of greater than 10^4 to 5×10^4 per g wet wt oyster^{-1} significantly reduced cell division rate, suggesting that a density dependent feedback mechanism is operative. Decreases in temperature and salinity of *in vitro* culture conditions also resulted in slower *P. marinus* growth rates (Gauthier and Vasta 1995, Dungan and Hamilton 1995, La Peyre 1996). Likewise, investigations of *P. marinus* activity in nature and in *in vivo* laboratory experiments have demonstrated the pronounced effect of temperature and salinity on *P. marinus* infection progression in oysters. Field obser-

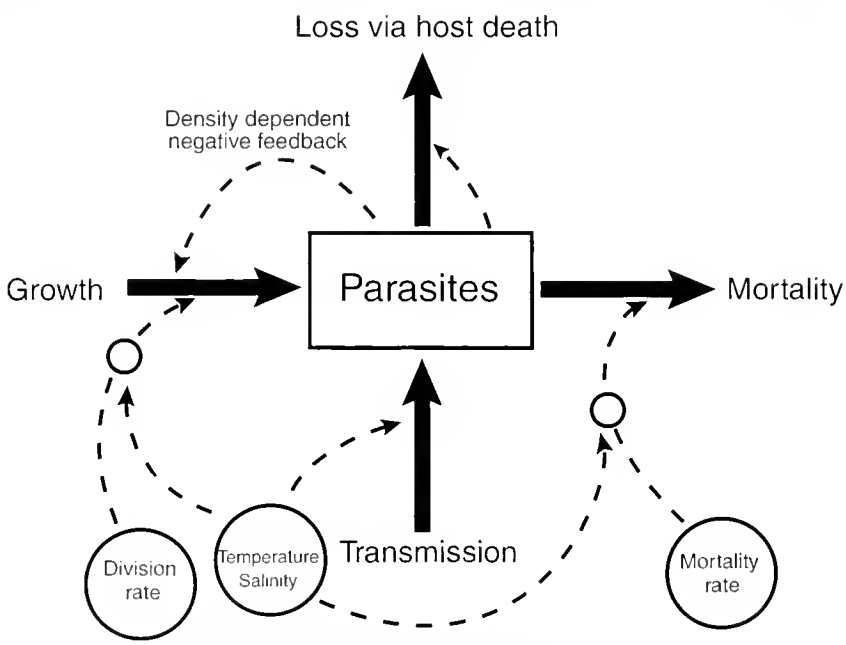

Figure 1. Schematic diagram of the *P. marinus* model.

TABLE 1.

Model forcing functions, state variables, flows, feedbacks and parameters.

Description	Symbol	Default values and/or unit
Forcing variables		
Temperature	$T(t)$	C
Salinity	$S(t)$	ppt
State variables		
Parasites	$P(t)$	cells (g wet wt)$^{-1}$ d^{-1}
Flows		
Parasite growth	$P_G(t)$	cells (g wet wt)$^{-1}$ d^{-1}
Parasite transmission	$P_T(t)$	cells (g wet wt)$^{-1}$ d^{-1}
Parasite mortality	$P_M(t)$	cells (g wet wt)$^{-1}$ d^{-1}
Parasite loss via host death	$P_L(t)$	cells (g wet wt)$^{-1}$ d^{-1}
Feedbacks		
Parasite density dependent self-limitation of $P_{dis}(t)$	$FB_p(t)$	
Parameters		
Parasite growth threshold density	A_p	10,000 cells (g wet wt)$^{-1}$ d^{-1}
Parasite maximum density	G_p	2×10^6 cells (g wet wt)$^{-1}$ d^{-1}
Parasite division rate	μd	d^{-1}
Parasite transmission rate as function of salinity	μt_{sal}	at S >15 = 1000 cells d^{-1} at 15 > S > 12 = 500 cells d^{-1} at 12 > S > 9 = 100 cells d^{-1} at 9 > S > 6 = 50 cells d^{-1} at S < 6 = 0 cells d^{-1}
Parasite mortality rate as function of salinity	μm_{sal}	at S > 12 = 0.0175 d^{-1} at 12 ≥ S ≥ 8 = 0.02 d^{-1} at 8 > S ≥ 3 = 0.0175 d^{-1} at S < 3 = 0.006 d^{-1}
Parasite loss via host death	μl	at P(t) >100,000 = 0.50 d^{-1}
Initial condition of state variable		
Parasite (WS Calibration)	P_0	801 cells (g wet wt)$^{-1}$
Parasite (WS Validation)	P_0	3927 cells (g wet wt)$^{-1}$
Parasite (HH)	P_0	537 cells (g wet wt)$^{-1}$
Parasite (DWS)	P_0	135 cells (g wet wt)$^{-1}$

vations indicate that parasite proliferation occurs at temperatures above 20°C (Andrews and Ray 1988). This observation has been supported by laboratory experiments in which infection progression was followed in artificially infected oysters held at 10, 15, 20, and 25°C (Chu and La Peyre 1993). Chu and La Peyre (1993) reported that only those oysters held at 20 and 25°C had moderate to heavy infections at the termination of the 7-wk experiment. Similarly, infection progression in oysters maintained in the laboratory at 25°C for a 6-wk period was delayed at 12 ppt and ceased at salinities below 9 ppt (Ragone and Burreson 1993).

In the model, parasite cell growth, the increase in parasite density as a result of cell division, is dependent on salinity, temperature, and parasite density. The model equation for cell growth was derived as:

$$P_G = P * \mu d * (1-((P-Ap)/(Gp-Ap)) \quad (2)$$

where P_G = parasite growth, P = parasite density (cells (g wet wt)$^{-1}$), μd = parasite division rate (d^{-1}), Ap = threshold parasite density (cells (g wet wt)$^{-1}$), and Gp = maximum parasite density (cells (g wet wt)$^{-1}$).

Since *P. marinus* growth has been demonstrated to be density dependent (Saunders et al. 1993, La Peyre 1996) a nonlinear density dependent negative feedback mechanism was incorporated into the equation for cell growth. Maximum density (Gp) was set at 2,000,000 parasites (g wet wt)$^{-1}$. This value was based on maximum *P. marinus* densities observed in oysters sampled from Wreck Shoal, James River, Virginia during a study conducted from 1993 to 1994 (Oliver et al. 1998). The maximum density represents the density at which space and or nutrients are so limited that parasite division no longer occurs. The threshold density (Ap), the density above which parasite growth is inhibited by intra specific competition for space and or nutrients was set at 10,000 (g wet wt)$^{-1}$. This value was also inferred from field data and represents an approximation of cell abundance in an oyster having a moderate intensity infection.

Parasite division rate (μd) is dependent on temperature and salinity. Field observations indicate that infections progress at temperatures exceeding 20°C (Burreson and Ragone Calvo 1996). On this basis, parasite division only occurs at temperatures exceeding 20°C. Temperature control follows a standard exponential form using a Q_{10} of 2.0 to calculate cell division at temperatures greater than 20°.

Initially the model was run using parasite division rates that were taken from the literature; however, initial model simulations resulted in unnatural population dynamics. Hence an alternative estimation of *P. marinus* division rate was derived. As an alternative, site specific division rates were derived from field data and laboratory studies. Since 1987, the Virginia Institute of Marine Science has been conducting an intensive survey program to monitor *P. marinus* prevalence and intensity at three oyster bars located in the upper the James River, Virginia. Oysters (n = 25) have been sampled monthly from Wreck Shoal (WS), Horsehead Bar (HH),

and Deepwater Shoal (DWS) (Burreson and Ragone Calvo 1996) (Fig. 2). These oyster bars are located along a salinity gradient with average salinities for the years 1987 to 1994 of 14 ppt at WS, 9 ppt at HH, and 7 ppt at DWS (Burreson and Ragone Calvo 1996). Sampled oysters were examined for *P. marinus* using a semi-quantitative assay described by Ray (1954). Using this method infections are categorized according to intensity and assigned a value from 1–7 for light to heavy intensities, respectively. The weighted prevalence (average infection intensity) can then be calculated yielding a single value that describes the average parasite cell density (Mackin 1962). For the purpose of calculating weighted prevalence for the VIMS data, infection intensities were assigned ranks as follows: 2 for light intensity, 4 for moderate intensity and 6 for heavy intensity. More recently a quantitative assay was developed for determining total parasite density in whole oysters (Choi et al. 1989). Whole oyster parasite densities were found to significantly correlate with Ray assay tissue ranks (Choi et al. 1989, Bushek et al. 1994). The regression equation presented by Bushek et al. (1994) ($y = 0.176 - 0.463x + 0.205x^2$, where y = log 10 *P. marinus* cells (g wet tissue weight)$^{-1}$ and x = infection rank) was used to convert VIMS oyster disease survey data from weighted prevalence to average parasite density (Fig. 3). Cell division rates were then determined using the calculated parasite densities from each station for the months May to June and June to July 1990 to 1993. During these months temperature is rising above 20°C and cell densities are generally low, so the rates determined are appropriate estimates for an initial division rate. Division rates were calculated using the natural log formula for population growth. The average division rates were respectively 0.009, 0.014, and 0.042 divisions d^{-1} at DWS, HH, and WS. Average salinity at the sites for the same period was 4.6 ppt at DWS, 7.1 ppt at HH, and 11.7 ppt at WS. Using data from a laboratory study presented by Ragone and Burreson (1993), the rate of division at 20 ppt was estimated to be 0.06 d^{-1}. A significant correlation was observed between salinity and the estimated division rates ($P = 0.013$, $r^2 = 0.973$), and the resultant regression equation ($y = -0.009 + 0.004x$, where x = salinity) was used as a first approximation to incorporate salinity control on μd into the

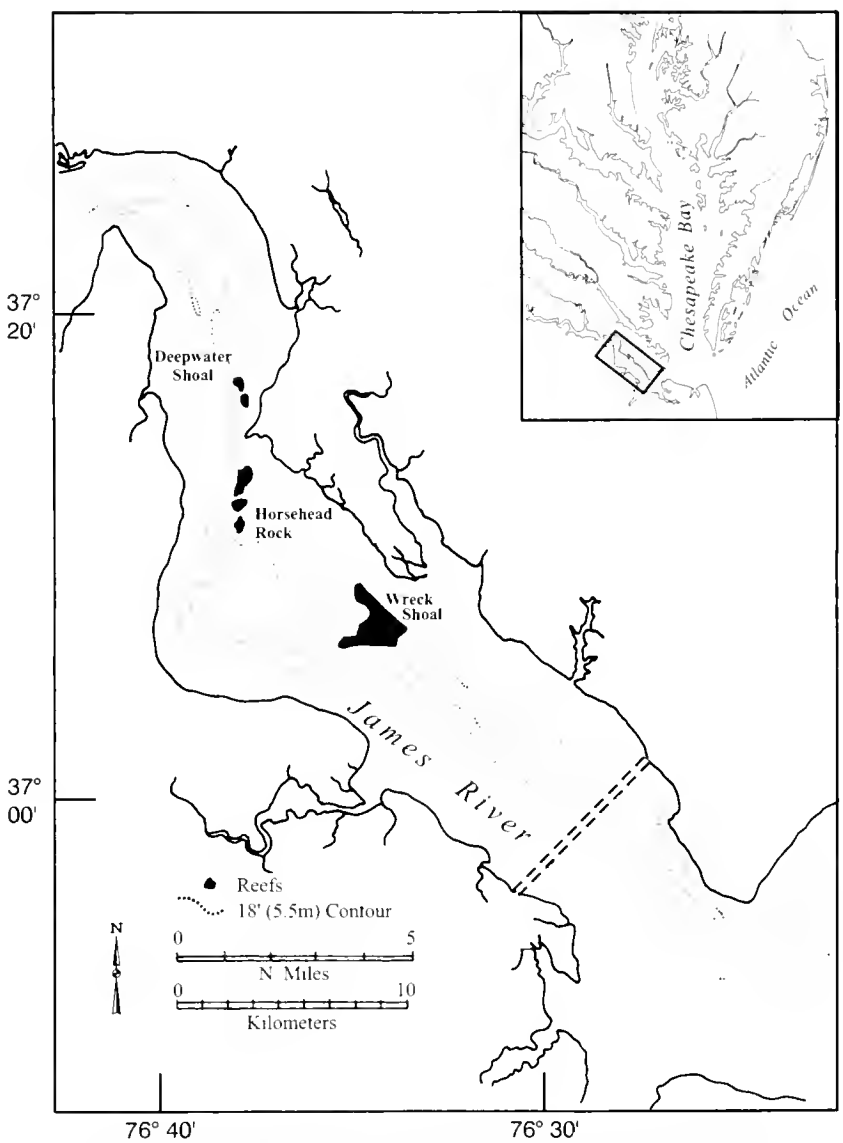

Figure 2. James River, Virginia oyster disease monitoring sampling sites.

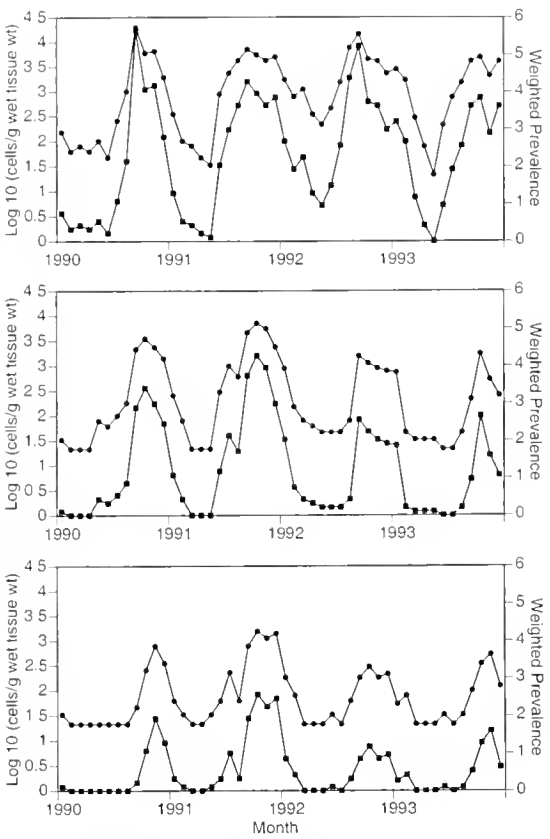

Figure 3. Conversion of *P. marinus* weighted prevalence (squares) to cells (g wet tissue wt)$^{-1}$ (circles). Converted 1990 to 1993 data is shown for three James River, Virginia oyster bars—Wreck Shoal (top), Horsehead (middle) and Deepwater Shoal (bottom). Conversions were calculated using equation presented by Bushek et al. 1994.

model. Sensitivity testing lead to refinement of this relationship and the final equation for parasite division was derived as:

$$\mu d\,(t) = (-0.009 + 0.0035 * S(t))\, e^{a\,(T(t)-20)} \qquad (3)$$

where $\mu d(t)$ = specific rate of parasite division (d^{-1}), a = Q10 conversion (0.06931), S = salinity (ppt), and T = temperature (°C).

Perkinsus marinus Death

Perkinsus marinus cell death is likely mediated by host-defense mechanisms and environmental conditions, but the processes involved are poorly understood. *Perkinsus marinus* is readily phagocytized by host hemocytes; however, it appears that these putative immunocytes do not readily destroy the parasite as observations of intrahemocytic destruction of *P. marinus* are limited (Anderson et al. 1995). Investigations on other mechanisms of the oyster immune system, including antimicrobial molecules, agglutinins, and lysins, have been conducted in relation to *P. marinus* but none have been demonstrated to be an effective control. Given the insufficient knowledge of the role of host defense in controlling *P. marinus*, no attempt was made to parameterize host-mediated cell death separately from other processes causing cell death. Instead, our approach was to characterize infection regression in general based on literature and on our own field studies. In the Chesapeake Bay, *P. marinus* abundance typically declines dramatically during the winter and spring. Andrews (1988) hypothesized that at temperatures less than about 20°C there is a physiological balance between *P. marinus* and the oyster, and host defense activities are favored over parasite proliferation. While it is clear that *P. marinus* multiplication is inhibited at low temperature (Chu and Greene 1989, Chu and La Peyre 1993, Chu and Volety 1997), it remains unclear as to whether host defense activities become favored within a particular temperature regime. Ford et al. (1999) examined Andrews' hypothesis by following *P. marinus* burdens in oysters maintained at 15°C and two different salinity treatments (12–14 ppt and 25–28 ppt) and found that oysters were unable to eliminate infections during the 11 week experiment. In contrast, they found that infections progressed during the study. While this study suggests that 15°C is not an optimum temperature for *P. marinus* elimination, it does not negate the possibility that a temperature lower than 15°C may be optimal for parasite elimination.

In an effort to gain a better understanding of *P. marinus* infection regression dynamics, monthly parasite elimination rates were determined from our 1990 to 1993 James River oyster disease survey data and examined in relation to temperature and salinity. Monthly *P. marinus* weighted prevalences were converted to parasite densities as described above, and parasite mortality rates were subsequently determined for the months October to November, November to December, December to January, January to February, and February to March. The parasite mortality rates were then examined in relation to average monthly temperature and salinity. Parasite mortality rate significantly correlated with temperature, increasing with decreasing temperature ($P < 0.0001$, $r^2 = 0.241$). On average the highest parasite mortality rates were observed at the higher salinity station, Wreck Shoal, than upriver at the lower salinity stations Horsehead Rock and Deepwater Shoal. Average salinities for Wreck Shoal, Horsehead Bar, and Deepwater Shoal for the months November through April 1990 to 1993 were respectively 12, 6, and 3 ppt.

Based on the field results described above, the model equation for parasite mortality was derived with both temperature and salinity controls. Since parasite mortality rates were higher at Wreck Shoal than at the two upriver stations, parasite mortality was set to optimally occur at 8 to 12 ppt. Within this salinity range the parasite is assumed to be relatively inactive while salinity is still high enough for host defense mechanisms to be operative. Base parasite mortality rates were altered with the highest rate occurring at 8–12 ppt, while slightly lower rates were set for >12 ppt and for 3–8 ppt. At <3 ppt, host physiological processes are assumed to be inactive, so parasite mortality rate was set extremely low. In the model parasite mortality occurs only at temperatures <18°C and >3°C. Between 18 and 3°C, temperature control of parasite mortality follows a standard exponential decay form using a Q10 of 2.0. Base rates were derived through sensitivity analysis of values within the range of those observed in the field. The equation for parasite mortality was derived as:

$$P_M = P * \mu m \qquad (4)$$

where P = parasite density (cells (g wet wt)$^{-1}$) and μm = specific parasite mortality rate (d^{-1}),

Such that:

$$\mu m\,(t) = \mu m_{sal} e^{a\,(18-T(t))} \qquad (5)$$

where $\mu m(t)$ = specific rate of parasite mortality; μm_{sal} = parasite mortality rate (d^{-1}) as a function of salinity where:

$\mu mt_{sal} = 0.0175$ at $S > 12$ and $8 > 3$ ppt,

$= 0.02$ at $12 \geq S \geq 8$ ppt and

$a = 0.003$ at $S < 3$ ppt;

a = Q10 conversion (0.06931), S = salinity (ppt) and T = temperature (°C).

Perkinsus marinus Transmission

Although it is well documented that transmission of *P. marinus* is direct from oyster to oyster, the natural dynamics of transmission are poorly understood. The prevailing conceptual model is that transmission occurs during periods of high oyster mortality as infective *P. marinus* cells are disseminated upon death and decomposition of infected oysters (Andrews 1988). Flow cytometric techniques have been developed that allow quantification of disseminated *P. marinus*-like cells in the water column (Roberson et al. 1993). This technique was employed to systematically examine the seasonality of *P. marinus* infection acquisition in oysters in relation to water column abundance of *P. marinus*-like cells, oyster mortality, and temperature (Ragone Calvo et al. 1995). Distinct peaks of all three parameters occurred during the month of August, following maximal summer temperatures (Fig. 4) (Ragone Calvo et al. 1995). Water column *P. marinus*-like cell abundance, infection acquisition, and oyster mortality decreased from summer maximums as temperatures decreased in September and October and remained at "wintertime" low levels from October through the termination of the study in March. These results support the currently accepted hypothesis that infective stages of *P. marinus* originate primarily from dying oysters and are most abundant in August.

Simulations were run both with and without transmission of parasites. When transmission is incorporated in the model, it occurs as a single event occurring once each year on Julian day 218. This timing corresponds with the early August transmission event observed by Ragone Calvo et al. (1995). In the model, the number of parasite cells transmitted is a function of salinity such that:

$$P_T = \mu t_{sal} \tag{6}$$

where μt_{sal} = the specific rate of parasite transmission (d^{-1}) as a function of salinity and has default values of:

1000 cells at $S > 15$ ppt,

500 cells at $15 > S > 12$ ppt,

100 cells at $12 > S > 9$,

50 cells at $9 > S > 6$ ppt and

0 cells at $S < 6$ ppt.

Salinity control of transmission is assumed to occur for two reasons: (1) infective cells are likely diluted by river discharge and (2) *P. marinus* infection levels and associated oyster deaths are limited in lower salinity areas. Clearly, this is an oversimplification of a process that is quite complex, but for lack of information it is a first approximation for model development and simulation analysis.

Parasite Loss via Host Death

It is assumed that the average *P. marinus* within-host abundance in any oyster population decreases as a result of the death of heavily infected hosts. This loss of parasites is incorporated into the model by the equation:

$$P_L = P(t) * \mu l \tag{7}$$

where μl = parasite loss rate (d^{-1}) and where $\mu l = 0.50$ at $P(t) > 100{,}000$ cells(g wet wt)$^{-1}$ and 0.00 at $P(t) < 100{,}000$ cells(g wet wt)$^{-1}$.

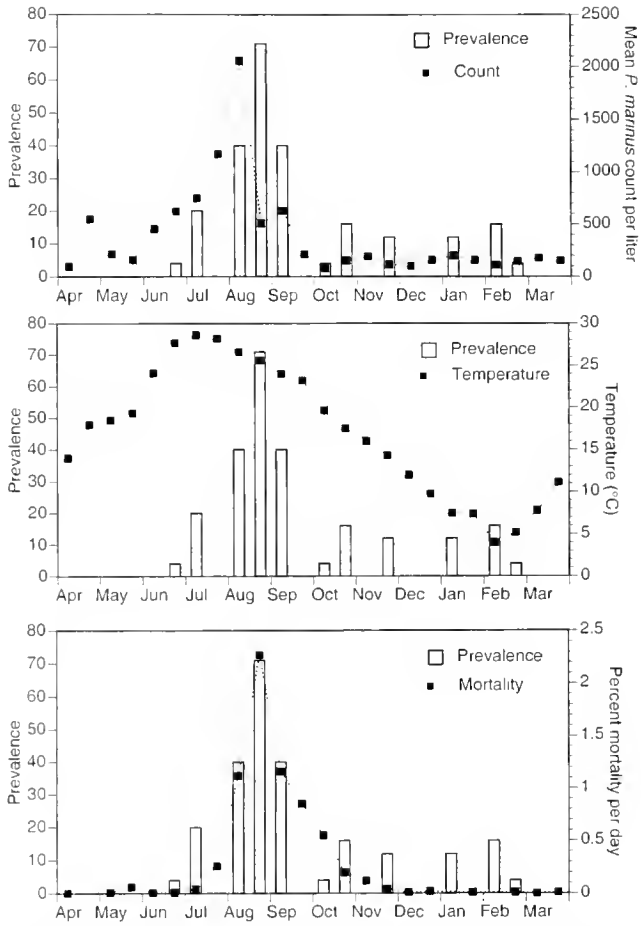

Figure 4. Results of *P. marinus* transmission study conducted by Ragone Calvo et al. (1995). *Perkinsus marinus* infection acquisition (prevalence) by previously uninfected sentinel oysters in relation to biweekly mean water column cell counts (top), biweekly mean temperature (middle), and oyster mortality rate (bottom) in the lower York River, Virginia.

Environmental Parameters

The model is driven by two environmental factors: temperature and salinity. The water temperature data series for both the model development data set (1990 to 1993) and the validation data set (1994 to 1999) were derived from a continuous monitor at the VIMS pier in the lower York River. Measurements were recorded at 6-min intervals; however, only daily means are utilized in the model. The salinity time series for Deepwater Shoal, Horsehead Rock, and Wreck Shoal were compiled from three sources—biweekly measurements taken by the State Water Control Board, weekly measurements (May through October only) taken in conjunction with the VIMS shellstring survey, and monthly measurements taken in conjunction with the VIMS oyster disease monitoring program. Monthly means for each of the three stations were

determined from the pooled data sets for incorporation into the model.

Model Calibration and Validation

As described above, since 1987, the Virginia Institute of Marine Science has been conducting an intensive survey program to monitor *P. marinus* prevalence and intensity at three oyster bars located along a salinity gradient in the upper the James River, Virginia. Separate portions of this data set were utilized for model calibration and validation. Data collected from Wreck Shoal during 1990 to 1993 was utilized for model calibration. The model was subsequently validated using 1994 to 1999 time series for Wreck Shoal, Horsehead Rock, and Deepwater Shoal. Since the monthly determinations of *P. marinus* prevalence and intensity were determined using Ray's fluid thioglycollate tissue assays (Ray 1954), which is only semi-quantitative, it was first necessary to convert the data to a quantitative format. This was accomplished using a conversion equation presented by Bushek et al. (1994) as described above. The conversion yielded average within-host abundances of *P. marinus* cells per gram wet tissue weight. Initial conditions for both model development and validation simulations were set to correspond to the actual *P. marinus* abundances that were observed in the first month of the time series (Table 1). *In vivo* parasite abundance was tracked at a daily time step.

Statistical Analysis

The significance of the association between predicted and actual *P. marinus* abundance was examined by linear regression using Abacus Concepts, Statview (Abacus Concepts, Inc., Berkeley, CA, 1992). Additionally, sensitivity analysis was conducted to examine the effect of variation of model parameters (μd, μm, μt, and μl) and input variables (S and T) on model performance. The rate parameters and input variables were systematically varied by ± 20% and sensitivity effects were assessed by calculating the residual, or root, mean square deviation (RMS) (Buzzelli et al. 1999) between the nominal model and the test cases, such that:

$$RMS = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(P_i - O_i)^2}$$

where P_i = model nominal (predicted) value, O_i = sensitivity test case value, and n = number of daily time steps in the six year simulation (n = 2190).

Differences between comparisons were normalized by calculating percent errors where the average RMS for the +20% and −20% test conditions is divided by the mean of O_i. The model is considered to be sensitive to parameters that yield % errors greater than 10%.

RESULTS

Model Calibration

The simulated within-host abundance of *P. marinus* using a lower York River water temperature time series and Wreck Shoal, James River salinity time series for the years 1990 to 1993 significantly correlated with actual observed abundance ($P < 0.0001$, $r^2 = 0.761$) (Fig. 5). The simulation generated a dynamic steady state in which parasite abundance exhibited a distinct seasonal periodicity with maximum abundance occurring in mid-August through mid-September following peak summer temperatures.

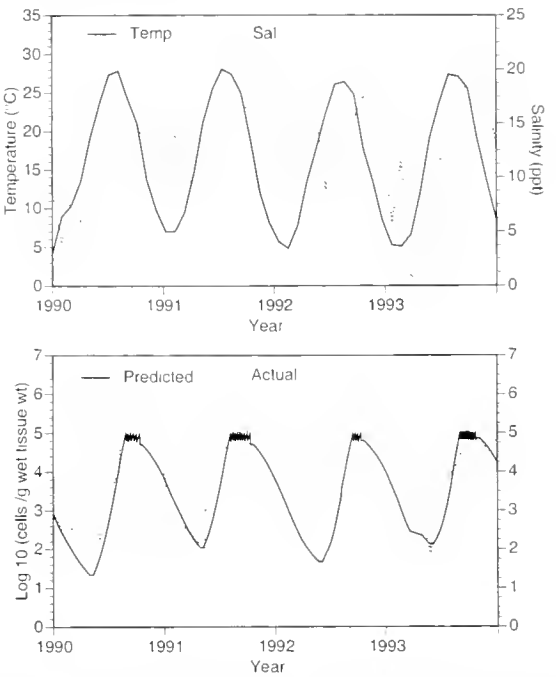

Figure 5. Simulation results for model development-time series, Wreck Shoal, James River, Virginia 1990 to 1993, showing temperature and salinity time series (top) and predicted versus actual observed *P. marinus* abundances (bottom).

Maximum cell division rates ranged from 0.09 to 0.11 divisions d^{-1}. These rates resulted in generation times from 6–7 days. Parasite abundance declined as temperature decreased during the fall and winter months, and minimums were observed from late April to mid-May. Deviations of the predicted abundance from the actual abundance were most evident in year one and year three at times of minimum and maximum abundance. In each year of the time series maximum *P. marinus* abundance exceeded 100,000 cells (g wet wt)$^{-1}$ and hence decreases in abundance associated with host death were observed. The occurrence of parasite loss due to host death appears as very short term oscillations in the simulation; population mean abundance is depressed due to parasite loss and then immediately increases as a result of parasite growth. Hence, the duration of the short-term oscillations yields information on the relative extent of parasite-associated host mortality from year to year.

The 1990 to 1993 environmental time series was quite variable. Annual salinity minimums and maximums ranged from 0.5 to 9.4 ppt and 15.7 to 19.1 ppt, respectively, while annual temperature minimums and maximums ranged from 4.2–6.9°C and 26.4–28.0°C, respectively (Fig. 5).

Model Validation

The model was validated using a lower York River temperature time series and site-specific salinity time series for Deepwater Shoal, Horsehead Bar, and Wreck Shoal, James River, Virginia for the years 1994 to 1999. The environmental time series included a broad range of conditions with unusually warm years (1997, 1998) and cold years (1996), as well as wet (1996, 1998) and dry years (1995, 1999). Annual temperature minimums ranged from 2.7 to 7.7°C, and annual temperature maximums ranged from 26.7 to 28.4°C (Figs. 6–8). Annual salinity minimums ranged from 2.0 to

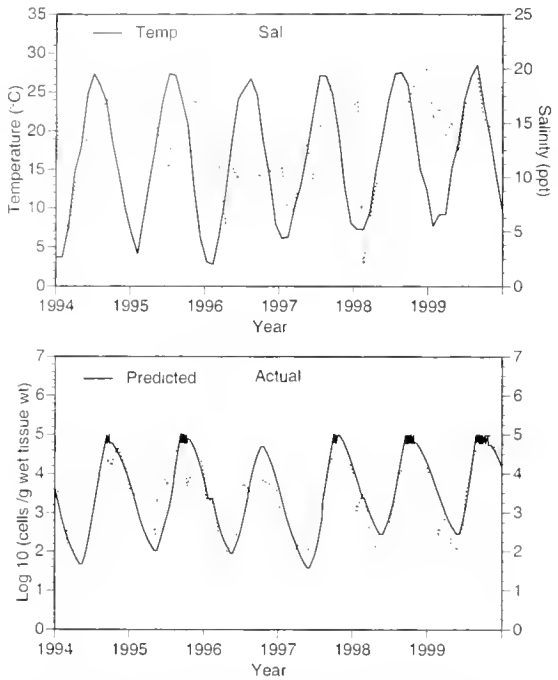

Figure 6. Validation model simulation results for Wreck Shoal, James River, Virginia 1994 to 1999 showing temperature and salinity time series (top) and predicted versus actual observed *P. marinus* abundances (bottom).

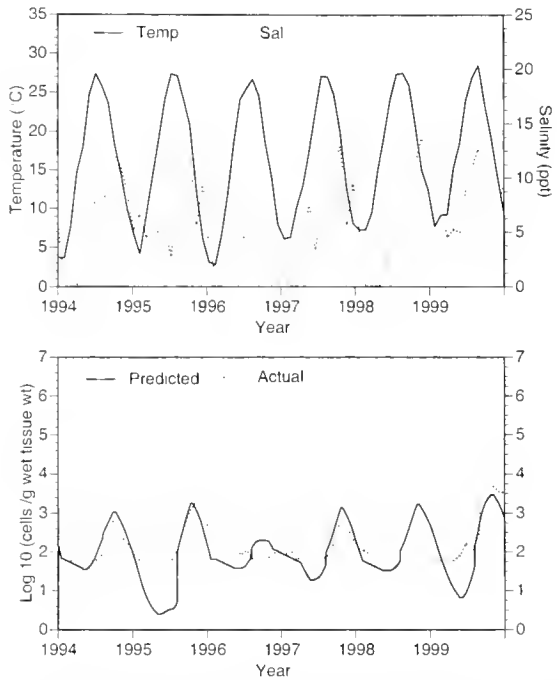

Figure 8. Validation model simulation results for Deepwater Shoal, James River, Virginia 1994 to 1999 showing temperature and salinity time series (top) and predicted versus actual observed *P. marinus* abundances (bottom).

12.5 ppt at WS, 0 to 5.5 ppt at HH, and 0 to 4.5 at DWS, while annual maximums in salinity ranged from 14.1 to 20.5 at WS, 7.3 to 16.0 at HH, and 6.2 to 13.7 at DWS (Figs. 6–8).

Predicted *P. marinus* abundance significantly correlated with actual observed abundance for all three sites; however, the strength of the correlation decreased in an upriver direction ($P < 0.0001$, $r^2 = 0.724$ at WS, 0.598 at HH, and 0.450 at DWS) (Figs. 6–8). As observed with the 1990 to 1993 time series simulations, simulations for all three oyster populations generated a dynamic steady state in which parasite abundance exhibited a distinct seasonal periodicity. Parasite abundance exponentially increased during the summer months reaching an annual peak in abundance between mid-September and mid-October. As temperature declined below 18°C during the fall, parasite abundance decreased. This decrease in parasite number continued through the winter and early spring reaching an annual minimum between late April and mid-May. Both the magnitude and timing of annual maximum parasite abundance varied both with respect to year and location. Annual abundance maximums were higher and generally occurred earlier at WS (Fig. 6) than at HH (Fig. 7) and at HH than at DWS (Fig. 8).

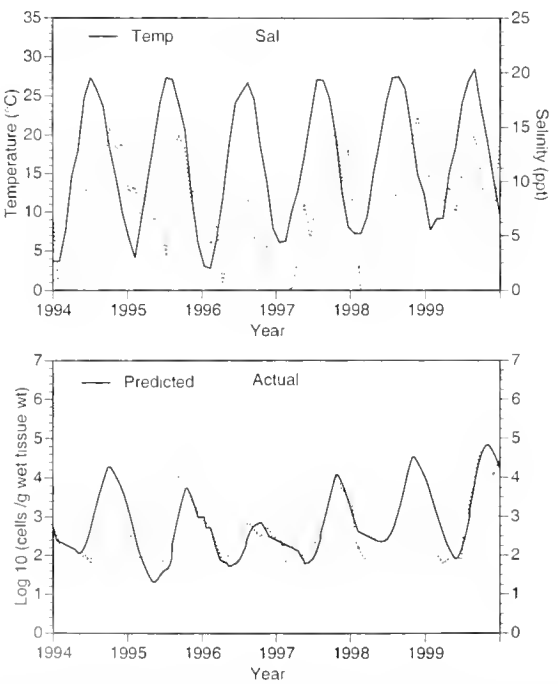

Figure 7. Validation model simulation results for Horsehead Shoal, James River, Virginia 1994 to 1999 showing temperature and salinity time series (top) and predicted versus actual observed *P. marinus* abundances (bottom).

The predicted annual periodicity of *P. marinus* abundance, particularly for WS, closely followed actual observed infection intensity and prevalence patterns in upper James River oyster populations. *Perkinsus marinus* abundance in the upper James River is typically highest during the months of September and October and lowest in May. Parasite abundance decreases in an upriver direction from WS to DWS and abundance peaks later in the upper river locations. For WS predicted annual averages, maximums and minimums in parasite abundance were always the same order of magnitude as actual observed abundance; however, at HH and DWS differences greater than 1 order of magnitude were sometimes observed. Specifically, at HH, 1998 predicted maximum abundance exceeded actual observed abundance by 1.03 orders of magnitude, and at DWS 1995 predicted minimum abundance were 1.38 orders of magnitude lower than actual observed values, and 1998 predicted abundance maximums exceeded actual abundance by 1 order of magnitude.

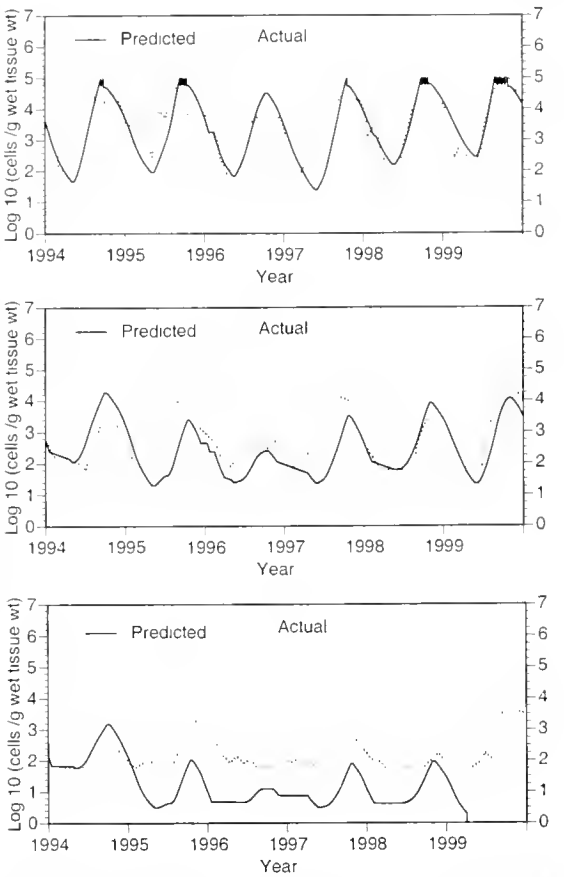

Figure 9. Model simulation results of predicted and actual observed *P. marinus* abundance for Wreck Shoal (top), Horsehead Rock (middle), and Deepwater Shoal (bottom) for the period 1994 to 1999 for condition in which annual transmission input is eliminated from model.

In the model *P. marinus* proliferation occurred only at temperatures greater than or equal to 20°C at salinity greater than 0 ppt. On average, these conditions were met, and cell division occurred 139 days per year. The number of days in which the parasite was active decreased in an upriver direction and inter annual variability was relatively high with the annual number of proliferation days ranging from 135–154 days at WS, 135–150 days at HH, and 90–150 days at DWS. Overall predicted mean, minimum and maximum cell division rates (d^{-1}) were respectively: 0.061 (± 0.017 SD), 0.023, and 0.104 at WS; 0.034 (± 0.017 SD), 0.0006, 0.073 at HH; and 0.026 (± 0.014 SD), 0.0001, 0.063 at DWS. The predicted division rates correspond to generation times ranging from 6 to 34 days.

Parasite death as incorporated into the model was modulated by both temperature and salinity. On average, parasite regression occurred 196 days per year. Predicted parasite mortality rates ranged from 0.006 to 0.057 d^{-1}. Average predicted parasite mortality rates (d^{-1}) were respectively 0.031, 0.023, and 0.021 at WS, HH, and DWS. Overall parasite regression rates were higher at WS than at HH and DWS; however, there were exceptions as both HH and DWS had higher parasite mortality rates than WS in 1995 and 1999.

Parasite loss via host cell death was incorporated into the model such that the state variable, parasite abundance, was reduced by 50% at parasite densities greater than 100,000 (g wet wt)$^{-1}$. At WS, parasite loss was an important regulator of parasite abundance and is evident in the simulations as high frequency short-term high oscillations in parasite abundance occurring coincidentally with summer abundance maximums. The periodicity of parasite cell loss due to host death at WS varied from year to year, being most apparent in 1999 and absent in 1996. Parasite burdens did not escalate to lethal levels at HH and DWS; hence loss via host death did not impact parasite abundance at these sites.

Parasite transmission was incorporated into the model as a single annual pulse of parasite cells into the host. The magnitude of the pulse is dependent on ambient salinity at the time. The number of cells transmitted ranged from 50 at salinity between 6 and 9 ppt to 1000 at salinity greater than 15 ppt. In the simulations transmission occurred in all years at all sites and varied from 50 to 100 at DWS, 50 to 500 at HH, and 500 to 1000 at WS.

In order to examine the relative importance of annual transmission events in maintaining *P. marinus* epizootics, the transmission component was eliminated from the model and simulations were run without the occurrence of an annual transmission event. The modified model produced simulations for WS and HH in which parasite abundance remained in a dynamic steady state (Fig. 9). Predicted *P. marinus* abundance for WS and HH correlated with actual observed abundance ($P < 0.001$, $r^2 = 0.709$ at WS, $r^2 = 0.444$ at HH); however, the correlation was not as strong as that produced by the original model. In contrast, *P. marinus* abundance for DWS did not maintain a dynamic steady state and parasite abundance gradually decreased over time with parasite extinction occurring in year 6 (Fig. 9).

Sensitivity Analysis

Sensitivity analysis was conducted in order to evaluate how relative changes in model parameters would effect model performance. The sensitivity of the model to key rate parameters varied with sampling location. For Wreck Shoal, the model was sensitive

TABLE 2.

Sensitivity analysis results for simulations for Wreck Shoal, Horsehead Rock, and Deepwater Shoal 1994–1999. Root mean square deviation (RMS) values are shown for +20% variation for each parameter.

Site	Parameter	+20%	−20%	Average RMS	% Error
Wreck Shoal	μd	0.18	0.23	0.21	5.7
	μm	0.42	0.48	0.45	13.3*
	μt	0.06	0.10	0.08	2.4
	μl	0.08	0.16	0.12	3.4
	S	0.19	0.42	0.30	9.2
	T	0.65	1.50	1.07	43.0*
Horsehead	μd	0.57	0.58	0.57	21.1*
	μm	0.55	0.76	0.66	22.8*
	μt	0.05	0.05	0.05	1.8
	μl	0.00	0.00	0.00	0.00
	S	0.76	0.80	0.78	29.9*
	T	1.16	1.48	1.32	64.5*
Deepwater Shoal	μd	0.28	0.35	0.32	15.9*
	μm	0.32	0.46	0.39	18.8*
	μt	0.07	0.08	0.07	3.7
	μl	0.00	0.00	0.00	0.0
	S	0.47	0.98	0.73	49.1*
	T	0.88	1.64	1.26	59.3*

* Sensitive parameters.

to only one rate parameter, parasite mortality, μm. A 20% change in μm resulted in a 13% error (Table 2). When model performance was assessed using the time series for Horsehead Rock and Deepwater Shoal, the model was sensitive to both parasite division rate and mortality rate and percent errors ranged from 16 to 23% (Table 2). The model was also very sensitive to temperature and salinity. Percent errors associated with a 20% change in temperature were 43% for Wreck Shoal, 66% at Horsehead Rock, and 59% at Deepwater Shoal. Salinity had a lesser effect than temperature. For Wreck Shoal, the model was not sensitive to 20% change in salinity, but for Horsehead Rock and Deepwater Shoal percent errors were relatively high, 30% and 49% respectively.

DISCUSSION

The model as currently constructed is simple but provides realistic population simulations for *P. marinus* within its host *C. virginica*. The dynamics of the populations described by these simulations reflects the importance of temperature and salinity in regulating *P. marinus* population dynamics. The annual cycle of parasite abundance exhibited a strong seasonal periodicity reflecting the controlling influence of temperature. As observed in nature (Andrews 1988, Burreson and Ragone Calvo 1996), simulated parasite abundance peaked approximately 2–3 months after annual maximums in temperature were observed. Under favorable salinity conditions parasite growth occurred at temperatures greater than 20°C, and parasite death occurred at temperatures less than 18°C. Hence, abundance increased from late spring until early fall and decreased from late fall to early spring.

The magnitude of parasite abundance maximums and minimums was modulated by salinity. *Perkinsus marinus* abundance progressively decreased with decreasing salinity. This pattern, which is the result of slower growth rates at low salinities compared to high salinities, is also observed in natural populations. *Perkinsus marinus* prevalence and infection intensity in oysters sampled along a salinity gradient in the upper James River, Virginia were correlated with salinity (Burreson and Ragone Calvo 1996). Summer and fall *P. marinus* infection intensities in oysters from the lower-most salinity areas were always light in intensity while moderate and heavy intensity infections were common in oysters collected from higher salinity areas (Burreson and Ragone Calvo 1996). Similarly, the abundance of overwintering parasites was higher in higher salinity areas than in lower salinity areas (Burreson and Ragone Calvo 1996).

Correlations between predicted parasite abundance and actual observed abundance were surprisingly strong, indicating that the main factors responsible for the regulation of *P. marinus–C. virginica* population dynamics in the upper James River, Virginia have been incorporated in the model. However, there remains room for improvement and some discussion regarding the deviation of predicted abundance from actual observed abundance. The strength of the correlation between the predicted and observed data decreased with decreasing salinity. This deviation may be in part due to two reasons. First, actual observed abundance data were calculated from a database that relied on a semi-quantitative diagnostic method. It is likely that some error was associated with the conversion of the semi-quantitative data to a quantitative format. The numerical ranks assigned (2, 4, and 6) to convert infection intensity scores (light, moderate, and heavy) to weighted prevalence were restrictive in setting lower and upper limits to the data. Much of the deviation observed in the DWS simulation is likely a result of this conversion. The model consistently generated lower minimum parasite abundance than that observed in the actual data; however, by assigning all infections that were categorized as light a ranking value of 2, the observed data minimums were most likely somewhat inflated. Second, there may be differences in parasite susceptibility between the three oyster populations. Wreck Shoal oysters, located farther down river, have likely experienced much stronger selection pressure from the parasite than Horsehead Rock and Deepwater Shoal oysters. That selection pressure may have affected parasite division and mortality rates, hence, parasite rates that were calibrated based on Wreck Shoal data may not be the best fit for the stations located farther upriver.

The results of sensitivity analysis indicated that several factors are important in determining model performance. The model was sensitive to salinity and temperature as well as to both parasite division and mortality rates. Interestingly, the degree of the model's sensitivity to these parameters varied with oyster bar. A twenty-percent change in the parameters had a lesser effect at Wreck Shoal than at Horsehead Rock and Deepwater Shoal. Since Horsehead Rock and Deepwater Shoal are located farther upriver than Wreck Shoal they more frequently experience salinity declines, which can be unfavorable for both parasite and host. It is likely that environmental thresholds affecting parasite and host activities are more frequently approached in the upriver locations. Estuarine environments experience large salinity and temperature fluctuations in relatively short time periods. In developing the model we derived our principal *P. marinus*-salinity relationships from a record of monthly field data that did not allow us to characterize the effect of shorter-term environmental fluctuations. Furthermore, it is likely that there are lag periods associated with initiation of parasite and host activities following the occurrence of a threshold condition. As presently constructed, lag times are not considered in the model. More detailed information about the environmental control of parasite division and mortality rates would improve model performance. This information would best be provided by experimental studies under controlled conditions.

Perhaps one of the most interesting results of the simulations was the fact that at WS and HH, a steady-state parasite abundance could be generated without the input of annual transmission events. Although parasite extinction ultimately occurred at DWS under conditions of no transmission, extinction took a period of nearly 6 years. These simulations suggest that a single transmission event may be sufficient for *P. marinus* to become enzootic within specific year classes of oysters in the moderate to high salinity areas, while periodic transmission events are required for the parasite to persist within specific year classes in low salinity areas. In the moderate–high salinity areas, parasite abundance would ultimately decrease as the pool of infected oysters is diluted by the presence of new recruits and the parasite would eventually become extinct when all infected hosts die. *Perkinsus marinus* became established in the upper James River oyster seed bed areas as a consequence of several consecutive drought years in the mid 1980s. Since that time the parasite has continued to be present in these historically disease-free areas. If the model simulations are correct, this would suggest that more than one transmission event has occurred in these low salinity areas. This clearly presents a very interesting area of focus for future research that could lead to the development of new management strategies. The current conceptual model of *P. marinus* transmission dynamics maintains that transmission occurs as infective cells are disseminated from dead hosts. Since there is little disease-associated mortality of oysters in

these low salinity areas, transmitted cells must be originating from live oysters or from downriver sources. Intensive harvesting of oysters from these downriver source areas, on a rotational basis, may be a means of preventing the recurrence of *P. marinus* transmission into the uppermost James River seed areas.

The outcomes of simulations generated by the model presented here are quite different from those presented by Powell et al. (1996). Powell et al (1996) developed a more complex model for *P. marinus* population dynamics coupled with oyster population dynamics in the Gulf of Mexico. Interestingly, their simulations were unable to generate an epizootic simply with changes in temperature and salinity. The outcomes of their simulations suggest that oyster populations must first be stressed by some other mechanisms before high temperature and high salinity conditions can facilitate epizootic oyster mortalities. They point to food availability and recruitment failure as two such mechanisms. Until the Chesapeake Bay model presented here is further developed to include host population dynamics the comparison with the Gulf of Mexico model remains problematic. However, it is interesting to note that results of simulations from both models indicate that once an epizootic is triggered it is very difficult to eliminate.

As currently constructed, this model provides realistic population simulations for *P. marinus* populations in the Chesapeake Bay. Development of the model has provided an excellent format for synthesizing data and for focusing attention on gaps in knowledge and research needs. Future development and evaluation of the model will yield a better assessment of thresholds for population maintenance and a better understanding of conditions that may lead to the termination of *P. marinus* epizootics and parasite extinction.

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A BIOCHEMICALLY BASED MODEL OF THE GROWTH AND DEVELOPMENT OF *CRASSOSTREA GIGAS* LARVAE

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ABSTRACT A biochemically based model was developed to simulate the growth, development and metamorphosis of larvae of the Pacific oyster, *Crassostrea gigas*. The model is unique in that (1) it defines larvae in terms of their protein, neutral lipid, polar lipid, carbohydrate, and ash content; (2) it tracks weight separately from length to follow larval condition index; and (3) it includes genetic variation in growth efficiency and egg quality to better simulate cohort population dynamics. The model includes parameterizations for larval filtration, ingestion, and respiration, which determine growth rate, and processes controlling larval mortality and metamorphosis. The initial biochemical content of the larva is determined by the composition of the egg. Changes in the initial ratios of protein, carbohydrate, neutral lipid, and polar lipid occur in response to the biochemical composition of available food as the larva grows. Modeling the process of metamorphosis requires a series of size-based and biochemically based triggers: (1) larvae become potentially competent to metamorphose at 275 μm, following a decrease in filtration rate at 250 μm; (2) larvae become competent to metamorphose when a daily decline in neutral lipid of 25% or more occurs; and (3) larvae metamorphose successfully if neutral lipid stores exceed polar lipid stores. Although based on simple biochemistry, the model succeeds in simulating such basic characteristics of *C. gigas* larval development and metamorphosis as larval life span and size structure at metamorphosis and the influence of egg and food quality and food quantity on survival. These results suggest that simple biochemical constructs may encompass the biochemical transitions most prominent in determining cohort success. Simulations of larval development show that for the smallest larvae, assimilation does not provide adequate resources to explain observed growth, although measured filtration rates would indicate otherwise. Egg lipid stores are needed to sustain the larva. The simulations also identify egg sizes in the range 37–73 μm to be viable, very similar to observations. Egg sizes outside this range are predicted to be nonviable due to lipid deficiencies in early larval life. Similarly, simulations identify upper and lower genetic limits on growth efficiency beyond which larvae cannot acquire sufficient neutral lipid stores to successfully metamorphose. As food supply declines, animals with high growth efficiencies are selected in the simulation. Low-protein food diets are predicted to increase larval survival. High-protein diets result in insufficient carbohydrate and neutral lipid to cover metabolic and storage needs. Thus, the influence of growth efficiency is nonrandomly distributed across egg size and respiration rate and the influence seems to be mediated in part by food quantity and to a larger measure by food quality.

KEY WORDS: *Crassostrea gigas*, larvae, Pacific oyster, growth, development model, biochemical, food

INTRODUCTION

For benthic species that have a planktonic larval phase of their life history, survivability of the larvae is the key determinant of recruitment to the adult population. Thus, much research has gone into identifying factors affecting the growth, development, metamorphosis, and settlement of larvae, especially for species with commercial value, such as the Pacific oyster, *Crassostrea gigas*.

Larvae of *C. gigas* undergo growth and development that is typical of bivalve larvae, with progression through D-shaped, umbo, and pediveliger stages (Fig. 1). The fraction of developmental time spent in each stage is variable and the rate at which the larvae progress through each is affected by local temperature, salinity, and food conditions (Helm & Millican 1977, Malouf & Breese 1977, Nascimento 1980, Gerdes 1983a, His & Maurer 1988, Nell & Holliday 1988; His et al. 1989, Pauley et al. 1988, Arakawa 1990, His & Seaman 1992, Robinson 1992, Thompson & Harrison 1992, Laing 1995, Thompson et al. 1996). The range of fractional development times reported for the small umbo to pediveliger stages (Fig. 1) is a reflection of these effects on larval growth.

Once spawned, the ultimate fate of *C. gigas* larvae is determined by the interaction of a number of factors. The first is the initial biochemical composition of the egg released by the adult, in particular the initial egg lipid content. Studies have shown correlations between egg size, lipid content, and bivalve larval development (Helm et al. 1973, Gallager et al. 1986, Gallager & Mann 1986a, Lee & Heffernan 1991). An implication of these studies is that initial egg lipid content is a determinant of larval survivorship and success at metamorphosis.

A second factor is the ability of the larvae to grow and develop so that total time spent in the plankton is minimized, thereby reducing exposure to mortality from predation. Because spawning pulses by adult populations, in nature, occur at widely variable times throughout the spawning season, larvae experience a widely varying set of environmental conditions. The timing of environmental conditions relative to a particular phase of larval life can greatly affect the total time needed for larvae to complete development (Dekshenieks et al. 1993). The concept of a critical period immediately post-hatch during which many planktonic larvae are particularly susceptible to low food supply is now well established (Cushing & Dickson 1976, Anger et al. 1981, Taggart & Leggett 1987), and the available data for bivalve larvae support the sensitivity of larvae to periods of low food supply (Gallager et al. 1986, His and Seaman 1992, Laing 1995). Studies also suggest that food quality, not just food quantity, is a critical issue (Thompson et al. 1994, 1996). Insufficient dietary lipid, for example, significantly limits larval growth and survival (Helm et al. 1973, Gallager et al. 1986).

A third factor affecting larval survival is the ability to acquire sufficient internal energy stores to successfully complete metamorphosis and set. Studies have shown that larvae can reach the size

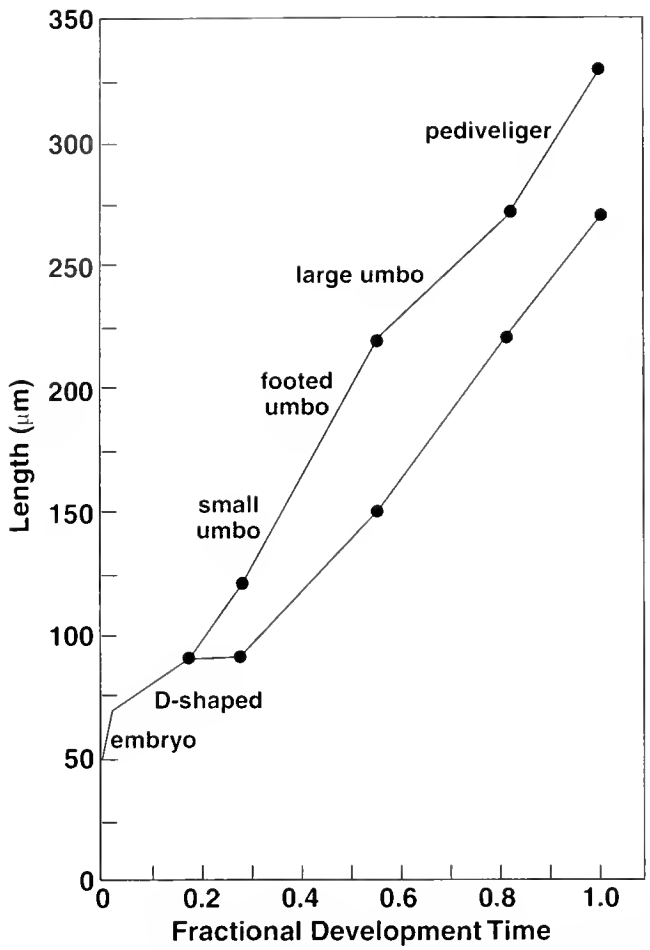

Figure 1. Schematic of the developmental stages of *Crassostrea gigas* larvae as a function of fraction of development time. The two curves bracket the range of sizes for the different stages, as reported in Arakawa (1990).

needed for metamorphosis but be unable to complete this step (Robinson 1992, Haws et al. 1993, Laing 1995). The possibility that a short-term deprivation of food early in larval life may reduce metamorphosis success can be inferred from a variety of studies that emphasize the critical importance of adequate food throughout larval life.

The increasing emphasis on the importance of an adequate diet, including quantity and quality, in determining a larval cohort's success and the recognition that adequate energy resources are needed for successful metamorphosis suggest the need to incorporate biochemical transfers into models of larval growth and development. The existing larval models determine growth from ingestion rate, decremented by the energy losses due to respiration and incomplete digestion (e.g., Carlotti & Sciandra 1989, Dekshenieks et al. 1993, 1996). These types of models, however, cannot be used to examine issues of food quality, nor can they be used to simulate the energy-reserve hypothesis underlying metamorphosis success. To investigate the influences of food quality and quantity on growth, development, and successful metamorphosis of *C. gigas* larvae, a biochemically based model was developed that includes explicit parameterizations for the metabolism of protein, carbohydrate, polar lipid, and neutral lipid within the standard parameterizations of energy flow via ingestion, assimilation, and respiration.

The following section provides a description of the *C. gigas* larval model and the parameterizations used in this model. This is followed by simulations that illustrate the effect of initial egg size, food quality, food quantity, and environmental conditions on larval growth, survival, and success at metamorphosis. The discussion section places these simulations within the context of the current understanding of the effect of environmental conditions and food quality on larval growth, survival, and metamorphosis.

MODEL DESCRIPTION

Model Structure and Governing Equation

The change in length for an individual larva over time is given by:

$$\frac{dL}{dt} = \alpha L \tag{1}$$

where L is larval length in μm and α is the rate at which the larva grows and has units of day^{-1}. Larval growth rate (α) is based on formulations that allow differential metabolism of the protein, carbohydrate, and lipid content of the food ingested by the larva. Thus, net production is expressed as the difference between assimilated ingestion (AI) and respiration (R):

$$NP_i = AI_i - R_i \tag{2}$$

where i represents the four basic biochemical components included in the model: protein, polar lipid, neutral lipid, and carbohydrate. An increase in larval length occurs when the sum of the four, $\sum_{n=1}^{4} NP_i$ is positive; when larval condition index is maximal for a given size; and when the restrictions imposed by certain biochemical ratios are simultaneously met. Thus, excess net production, ENP, is the basic quantity responsible for larval growth.

The specification of ENP, which determines α, is based on filtration, the metabolism of carbohydrates, polar lipids, neutral lipids, and proteins within the larva, and the conversion of the metabolized food into structural components of the larva versus the conversion into storage material. A basic assumption in this model is that the formation of structural components determines the increase in larval length. Material converted into storage components (i.e., neutral lipids) does not result in increased larval length. The conversions and parameterizations used to model these processes are described in the following sections.

The *C. gigas* larval model given by Eq. (1) was solved numerically using a third-order Adams-Bashforth scheme (Canuto et al. 1988) with a time step of 0.1 day.

Model Parameterizations

Observations from field and laboratory studies were used to derive the relationships that describe the processes affecting net production of *C. gigas* larvae. The basic units used in the model are grams, joules, and μm, and these are not necessarily always consistent with the units used for measurements. Thus, the first step in developing the larval model was to obtain conversions that allow the model calculations and output to be consistent with measurements and to be compared with observations. Also, many of the larval processes vary in amplitude or even form with larval size, requiring that relationships used to describe these be size dependent.

Length-to-Dry Tissue Weight Conversion

Numerous field and laboratory data sets exist that can be used to derive a length-to-dry tissue weight relationship for *C. gigas* larvae. However, the reporting of these data sets is quite variable, with some reported in terms of dry tissue weight (Gerdes 1983a) and others reported in terms of whole animal weight (His & Maurer 1988, Nascimento 1980, Waldock & Nascimento 1979). Dry tissue weight is the desired unit for deriving this relationship, so the data sets reported as whole animal weight were multiplied by a factor of 0.25, which corrects for the shell being 75% of the animal weight (His & Maurer 1988). The resultant data set (Fig. 2) was used to obtain the length-to-dry tissue weight relationship:

$$W = aL^b \tag{3}$$

where W is larval weight in ng and L is larval length in μm. The coefficients a and b are given in Table 1. The correspondence between Eq. (1) and the data sets is shown in Figure 2.

Length-to-Ash Weight Conversion

His and Maurer (1988) provide measurements of the percent of total dry weight as a function of larval size. These values were obtained by summing the percent organic matter represented by larval protein, carbohydrate, and lipids that were determined independently. As a comparison, His and Maurer (1988) also determine the percent total dry weight of organic matter by combustion of the whole animal. These data form the basis for developing a relationship to relate larval length to ash weight.

Comparison of the organic matter values determined by the sum of the components and those obtained from the heating method differ by about 5%. The difference was assumed to be due to protein, which is more completely measured by the heating method, so this difference was added to the protein value for a given size larva. The percent total dry weight for the largest larval size measured by His and Maurer (1988), 274 μm, was reduced from 29.1% to 26.2% because attempts at using the higher value to produce a relationship between larval length and ash weight gave unrealistic results. The reduction in percent total dry weight was obtained by maintaining the same proportional difference in the heating and sum of component determinations of percent dry weight as reported for other larval sizes. The rationale for the reduction in value is that the measured value included effects of metamorphosis that are not explicitly included in the larval model. The percent total dry weight for the 274 μm larva was also applied to the measured dry weight (7000 ng) for 320 μm larvae to provide an additional data point at the larger size.

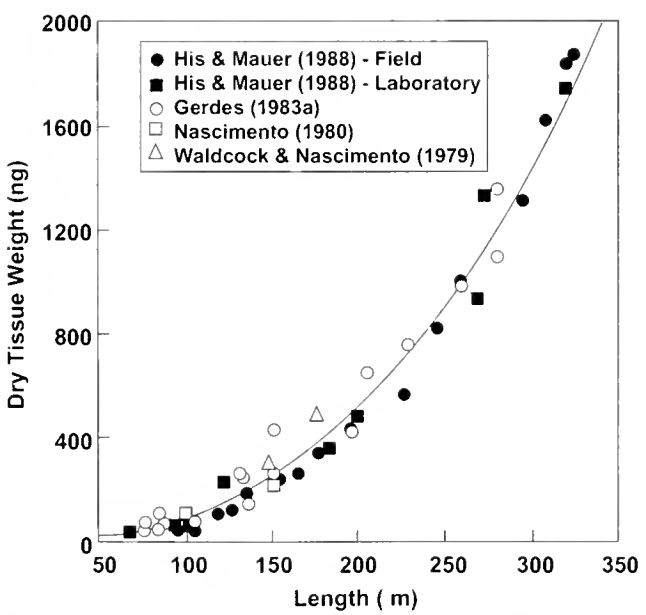

Figure 2. Dry tissue weight as a function of *Crassostrea gigas* larval length.

TABLE 1.

Definition, units, and values for the variables used in the *Crassostrea gigas* larva model equations.

Variable	Definition	Units	Value
a	constant	ng	6.745×10^{-4}
b	constant	none	2.557
c	constant	ng larva^{-1}	3.931×10^{-3}
d	constant	none	2.440
a_1	constant	none	0.3436
a_2	constant	μm^{-1}	−0.00347
a_3	constant	μm^{-2}	2.0323×10^{-5}
a_4	constant	μm^{-3}	-3.1107×10^{-8}
FR_o	constant	1 day^{-1}	24×10^{-6}
b_1	constant	none	−2.0909
b_2	constant	μm^{-1}	0.0335
b_3	constant	μm^{-2}	-3.2798×10^{-5}
c_1	constant	none	96.9948
c_2	constant	μm^{-1}	−1.1011
c_3	constant	μm^{-2}	4.3×10^{-3}
c_4	constant	μm^{-3}	-5.5985×10^{-6}
γ	constant	none	0.0025
α	constant	none	0.35
β	constant	none	0.08
L_o	initial larva length	μm	calculated
L_f	larva length	μm	100
δ	constant	none	0.5
L_s	larva length	μm	0.5
λ	maximum neutral lipid usage	% day^{-1}	75.
SL	upper larval size for neutral lipid use	μm	80.
r_o	base respiration rate	KJ day^{-1}	calculated
θ	constant	none	0.96
m_o	daily mortality rate	day^{-1}	
ES_o	central egg size	μm	50
$Resp_o$	central respiration rate	KJ day^{-1}	1.046
$2sd_{egg}$	standard deviation, egg size	μm	0.2
$2sd_{resp}$	standard deviation, respiration rate	KJ day^{-1}	0.2

The length-to-ash weight data set was fit with a regression of the form

$$AW = cL^d \tag{4}$$

where AW is larval ash weight in ng larva^{-1} and the coefficients c and d are given in Table 1. The correspondence between the data and fitted curve is shown in Figure 3.

Length-to-Protein-to-Ash Ratio Conversion

The protein values for the different sized larva reported in His and Maurer (1988) were divided by the corresponding larval ash

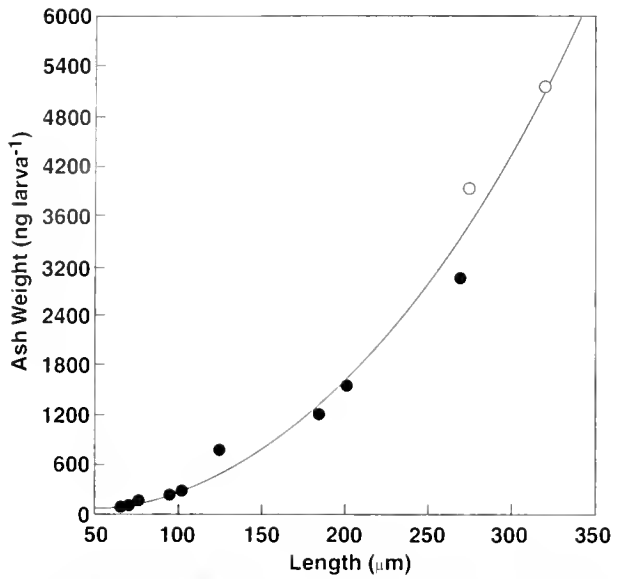

Figure 3. Ash weight as a function of *Crassostrea gigas* larval length. The solid circles are measurements given in His and Maurer (1988). The open circles represent measurements given in His and Maurer (1988) that were modified as described in the text.

weight values, determined as described in the previous section, to obtain protein-to-ash ratios as a function of larval size (Fig. 4). The curve fit to these data is of the form:

$$PAR = a_1 + a_2 L + a_3 L^2 + a_4 L^3 \quad (5)$$

where *PAR* is the protein-to-ash ratio and the coefficient values are given in Table 1. This relationship describes a ratio that decreases from a value of 0.27 to a minimum of 0.18 at a larval size of about 100 μm, after which it again increases. The initial decrease is associated with the development of shell. The increase in the ratio as the larva gets larger reflects the decrease in the surface-to-volume ratio of the larva as it grows.

Carbohydrate, Protein, Polar Lipid, and Neutral Lipid Conversions

In the model, conversions between carbohydrate, lipid, and protein were needed to account for the debiting of the biochemical pools to cover the metabolic cost of respiration and the movement of carbon between different tissue types. The needed conversions are of two types. Those for respiratory demands are expressed in terms of energy (e.g., joules); those for tissue conversions are expressed in terms of moles carbon.

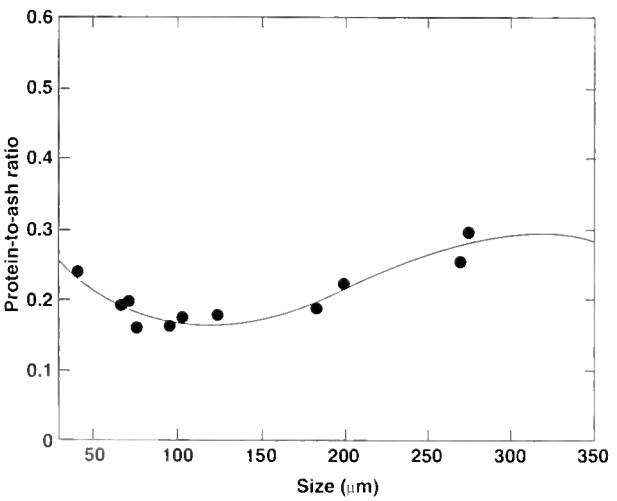

Figure 4. Protein-to-ash ratio as a function of *Crassostrea gigas* larval length. The solid circles are the ratios calculated from the protein and ash values given in His and Maurer (1988).

Respiratory demands were converted to equivalent energy values using 39.5 kJ g^{-1} for lipids, 23.6 kJ g^{-1} for protein, and 17.2 kJ g^{-1} for carbohydrates. These conversions are based on data given in Mann and Gallager (1985), Trytek and Allen (1980), and Crisp (1971).

Interconversion of biochemical constituents was based on carbon equivalent weights using palmitic acid, serine, and glucose residues, respectively, to define the ratio between moles carbon and molecular weight. In this way, carbon atoms were conserved, but the total constituent weight changed depending upon the contribution of carbon to the molar weight. So, for example, the conversion of tissue carbohydrate to tissue lipid is based on a ratio of 1.929, which expresses the relative weight of lipid and carbohydrate based on the equivalency of carbon atoms.

In addition, certain weight ratios between structural constituents were associated with healthy larvae. When in sufficient quantity, assimilated food constituents were allocated to tissue pools using these ratios. Deviations in the resulting tissue composition from these ratios resulted in mortality. The relationship between tissue lipid and protein was obtained from His and Maurer (1988), under the assumption that the total lipid content of *C. gigas* larvae, like *C. virginica* larvae, is about evenly split between neutral and polar lipids (Gallager et al. 1986, Whyte et al. 1987). This yielded a preferred tissue polar lipid-to-tissue protein ratio of 0.11 for healthy larvae. Information from the same sources was used to establish the equivalent ratio between tissue carbohydrate, most of which was assumed to be structural since neutral lipid is the primary storage constituent, and tissue protein. The value of the tissue carbohydrate-to-tissue protein ratio was set at 0.01. Both ratios were independent of larval size.

Parameterization of Processes

Filtration Rate and Filtration Efficiency

Filtration is the basic process by which *C. gigas* larvae obtain food. Therefore, accurate representation of this process is needed for larval growth to be correctly simulated. Gerdes (1983a) presents measurements of filtration rates for *C. gigas* at a range of food concentrations and larval sizes. These data show three distinct phases of larval filtration (see Fig. 8 in Gerdes 1983a). Initial filtration rates are low until the larva reaches about 100 μm, after which filtration rate increases exponentially to its maximum as the larva reaches 250 μm. At this size, larval behavior changes as the larva nears metamorphosis and filtration rate decreases dramatically.

The filtration rate observations given in Gerdes (1983a) were used to derive empirical relationships that provide the basic filtration structure of the model (Fig. 5):

$$FR = FR_o e^{(b_1 + b_2 L + b_3 L^2)}, \quad \text{for larva} \leq 250\ \mu\text{m} \quad (6)$$

$$FR = FR_o e^{(c_1 + c_2 L + c_3 L^2 + c_4 L^3)}, \quad \text{for larva} \geq 250\ \mu\text{m} \quad (7)$$

where *FR* is filtration rate. The coefficients in Eqs. 6 and 7 are given in Table 1.

Initial simulations that used the above relationships for larval

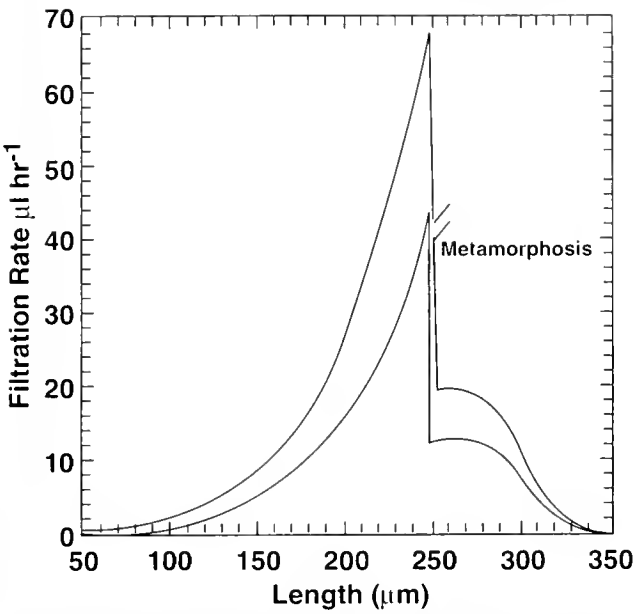

Figure 5. Upper curve, filtration rate as a function of *Crassostrea gigas* larval length calculated from Eqs. 6 and 7 as described in the text. Lower curve: filtration rate after corrections described by Eqs. 8 and 9 (for food concentration of 2 mg L^{-1}).

filtration rate resulted in growth rates that were too high. Reduction of the growth rate was accomplished by adding a factor to the filtration rate that reduces ingestion efficiency. This factor is of the form:

$$IE = \frac{\gamma}{Food}\left[\alpha + \beta\left(\frac{L - L_o}{L_f}\right)\right] \qquad (8)$$

where ingestion efficiency (IE) is a nondimensional quantity that depends on the ambient food concentration ($Food$) and larval size. Coefficient values are given in Table 1. The relationship given by Eq. 8 results in reduced feeding efficiency for all larval sizes, but with the maximum reduction associated with smaller larvae.

The rationale for reducing filtration efficiency is that the vellum is a multipurpose organ, so there must be some inefficiency in each of the activities and functions of this organ; otherwise the larvae could only swim at the rate that allows maximum ingestion and dispersal, and escape capabilities would be compromised. Thus, filtration rates measured in a laboratory setting for *C. gigas* larvae should be regarded as measures of vellum activity and not as measures of ingestion. In addition, most research has been conducted using saturating food concentrations. This would exacerbate any tendency for more food to be filtered from the water column than could be ingested by the larva.

Applying the ingestion efficiency factor (Eq. 8) to Eqs. 6 and 7 resulted in realistic larval growth rates, except for larvae smaller than 80 µm. Early in larval life the rapid changes leading to the development of the organs for feeding and digestion should further limit ingestion and/or assimilation efficiency. Thus, the filtration rate for small larvae (FR_s) was further reduced by:

$$IE_s = IE \cdot \delta \cdot \left(1 + min\left(1., \left(\frac{L - L_o}{L_s - L_o}\right)\right)\right) \qquad (9)$$

The above approach is based on the assumption that the vellum in small larvae is very inefficient at capturing food particles and/or that digestion is less efficient in small larvae. Coefficient values and definitions are given in Table 1.

A relationship between low food supply and increased feeding efficiency due to enlargement of the vellum (Strathmann et al. 1993) was not included in the model because the influence of vellum enlargement on filtration rate is unknown. The model may underestimate growth rate at low food supply.

Temperature and Salinity Effects

As with most bivalve larvae, the metabolic processes controlling growth in *C. gigas* larvae are affected by temperature and salinity (Lee & Lee 1968, Helm & Millican 1977, Ventilla 1984). His et al. (1989) provide measurements of larval growth rate for salinities between 20‰ and 35‰ and temperatures between 15°C and 30°C. These measurements start with larvae with a mean shell length of 57 µm, which is prior to the development of the D-shaped stage (Fig. 1), and extend through the first seven days of larval growth. The differences in the measured larval growth, for the fed larvae, between day 0 and day 7 were calculated for each temperature and salinity. The resultant values were linearly interpolated to obtain growth rates at intermediate temperature and salinity values. Normalizing this matrix of growth rates to the growth rate at 25°C and 30‰ provides the fractional change in larval growth rate at a given temperature and salinity (Fig. 6).

The measured growth rates given in His et al. (1989) were extended to the entire range of temperature encountered by *C. gigas* larvae by assuming that larval growth rate decreases in a linear fashion to zero between 15°C and 0°C and to zero between 30°C and 35°C. This pattern in growth rate is based on observations of increased larval abnormalities in these temperature ranges (Arakawa 1990). Similarly, for salinity, the growth rate at 20‰ was linearly reduced to zero at 10‰, after which growth rate remained zero. This was based on observations of increased larval abnormalities at low salinities (Arakawa 1990). For salinities above 35‰, larval growth rate at 40‰ was assumed to be one-half

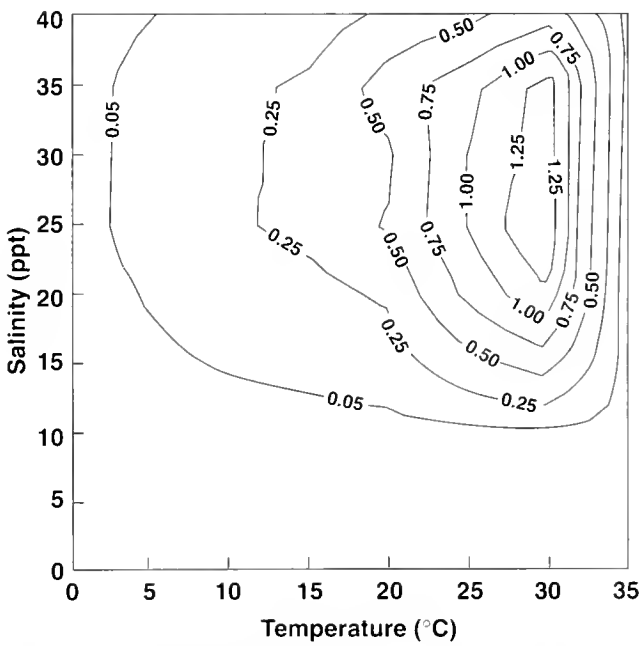

Figure 6. Fractional change in *Crassostrea gigas* larval growth rate as a function of temperature and salinity.

the larval growth rate at 35‰, and growth rates at intermediate salinities were obtained by linear interpolation. The assumption of reduced growth rate at 40‰ is based on measurements (Nell & Holiday 1988) that show *C. gigas* growth rate at 39‰ being one-half the value at 35‰.

The fractional change in larval growth over the entire range of temperature and salinity was verified by comparing simulated larval length with reported larval lengths measured at known temperatures and salinities (Helm & Millican 1977, Nell & Holiday 1988, His et al. 1989, Robinson 1992). These comparisons showed excellent agreement between simulated and observed larval length, except for lengths between 150 and 200 μm. At these sizes, mismatches of 10 to 45 μm occurred for the higher temperature and salinity values. Larvae of this size are growing rapidly at salinities above 24‰ and temperatures above 25° C, and hence the growth curves are steep. Therefore, slight mismatches in the reporting of measured length for a given larval age can greatly affect comparisons with simulated lengths.

Food Composition and Assimilation Efficiency

The *C. gigas* larval model allows for differential metabolism of protein, carbohydrate, polar lipid, and neutral lipid. For this to occur, the food ingested by the larva must be expressed in terms of the relative contribution of each of these biochemical constituents. Based upon measurements reported in Utting (1986), Roman (1983), and Lee et al. (1971), the average biochemical composition of marine algae, in terms of ash-free dry weight, was taken to be 3 parts protein, 2.5 parts carbohydrate, 0.6 parts polar lipid, and 0.4 parts neutral lipid. This basic structure defines the food reservoir for the larvae, for most simulations.

Handa (1969) provides assimilation efficiencies for plant material of 1.0 for protein, 1.0 for polar and neutral lipids, and 0.2 for carbohydrates. The reduced assimilation efficiency for carbohydrates arises because 80% of plant carbohydrate is structural or β-linked carbohydrate (e.g., the refractory portion) that cannot be digested by animals and is therefore not available as food. The available 20% represents labile carbohydrate. Multiplication of these assimilation efficiencies with the corresponding food fraction gives an overall assimilation efficiency for *C. gigas* larvae of about 0.7, which is within the range expected for bivalve larvae (estimated from growth efficiency by Jorgensen 1952).

Initial simulations showed that the above assimilation efficiencies resulted in growth rates for larvae less than 80 μm, which were too small. Observations show a drawdown of neutral lipid reserves during early larval life in *C. gigas* and *C. virginica* (Gallager et al. 1986, Gallager and Mann 1986a, Whyte et al. 1987, His & Maurer 1988), presumably to fill the carbon needs not covered by feeding. In the model, the early life stages of the larva were allowed to use neutral lipid stores to form structural material in the body. This was done by calculating a small larva factor (SLF_i) of the form:

$$SLF_i = max\left[0., \lambda\, \Delta t \left(\frac{SL - L}{SL - L_o}\right)\right] \qquad (10)$$

where i indicates protein, carbohydrate, polar lipid, or neutral lipid. This relationship calculates the proportionate length change for larva smaller than 80 μm in a given time increment (Δt), and the neutral lipid reserves are then used in proportion to the carbon requirement needed to sustain the change in length. The maximum neutral lipid that is used, given by λ, occurs when larvae are at their initial size, L_o. This amount decreases proportionately as the larva grows and becomes increasingly capable of feeding, and is zero at 80 μm. The mobilized neutral lipid is then converted into equivalent protein, carbohydrate, and polar lipid using the biochemical conversions given previously. This is the only instance in the model where protein is created *de novo*, rather than being obtained from food.

Thus, the assimilated ingestion (AE_i) can be expressed as the product of the filtration rate (FR), the ingestion efficiency (IE_s), temperature and salinity effects ($TSfactor$), food ($Food_i$), the assimilation efficiency (AE_i), and the small larvae factor (SLF_i) as:

$$AI_i = FR \cdot IE_s \cdot TSfactor \cdot Food_i \cdot AE_i \cdot SLF_i \qquad (11)$$

Fate of Assimilated Ingestion

The assimilated ingestion obtained from Eq. (11) is parameterized in terms of protein, neutral lipid, polar lipid, and carbohydrate, and the fate of each of these biochemical constituents differs within the larva (Table 2). Protein assimilated in a given time interval has, as its primary destination, the somatic protein pool. Protein may also be used to cover a respiratory deficit (discussed below) in accordance with the appropriate protein-to-carbohydrate-to-polar lipid ratio.

The carbohydrate needs of the larva are determined by the amount needed to maintain tissue carbohydrate in its proper proportion and that needed to cover the metabolic process of respira-

TABLE 2.

Destination of assimilated protein, carbohydrate, polar lipid, and neutral lipid in *Crassostrea gigas* larvae.

Food constituent	Primary destination in larva	Food deficit response	Food surplus response	Tissue maintenance deficit	Early life (<80 μm)
Protein	somatic P	NA	NA	respiration (P:C:PL)	NA
Carbohydrate	somatic C & respiration (P:C)	somatic PL (P:PL)	neutral lipid reserve	respiration (P:C:PL)	NA
Polar lipid	somatic PL (P:PL)	somatic C (P:C)	neutral lipid reserve	respiration (P:C:PL)	NA
Neutral lipid	neutral lipid reserves	somatic C (P:C); somatic PL (P:PL) respiration	NA	somatic C (P:C); somatic PL (P:PL); respiration	somatic C; somatic PL; somatic P; respiration

The particular biochemical ratio determining the conversion to individual reservoirs is indicated. Table columns two, three, and four indicate the fate of the food; column five indicates the fate of the tissue. Transfers of food that do not occur in response to deficit or surplus conditions are indicated by NA. Protein, carbohydrate, and polar lipid are indicated by P, C, PL, respectively.

tion (Table 2). Assimilated carbohydrate is the primary means by which larval respiratory needs are met (Table 2). The required somatic carbohydrate is determined so as to maintain the carbohydrate-to-protein ratio (0.01), and this amount is debited from the available assimilated carbohydrate and added to the carbohydrate pool. Excess carbohydrate (food surplus response in Table 2) becomes part of the larval neutral lipid reserve. When tissue imbalances occur (e.g., insufficient polar lipid to meet the tissue compositional requirements of the larvae), somatic carbohydrate is used to maintain larval polar lipid in its proper proportion.

The primary destination of assimilated polar lipid in the larva is the somatic polar lipid pool in accordance with the protein-to-polar lipid ratio (Table 2). Excess assimilated polar lipid goes to the larval neutral lipid pool. When carbohydrate imbalances occur, polar lipid reserves are mobilized to produce somatic carbohydrate in an amount that is consistent with maintaining the protein-to-carbohydrate ratio. Polar lipids are also used to cover tissue maintenance deficits arising from respiratory demands.

The primary destination of assimilated neutral lipid is the neutral lipid pool (Table 2). This internal pool is mobilized to maintain somatic carbohydrate and somatic polar lipid pools in accordance with the appropriate ratios when assimilated protein, carbohydrate, and polar lipid are not present in the proper proportions in the food. The neutral lipid pool can also be used to cover respiratory needs during periods of carbohydrate deficit. This pool also provides a means for small larvae, less than 80 μm, to produce somatic carbohydrate, polar lipid, and protein as well as cover respiratory costs early in larval life.

At any point in the development of the larva, the inability to maintain one of the biochemical constituent ratios or the inability to remove a deficit in a biochemical pool results in death of the larva.

Respiration

Respiration provides the only metabolic loss of assimilated energy in the *C. gigas* model. Measurements show that respiration rate for *C. gigas* larvae increases with larval size and with temperature (Fig. 7). Laboratory measurements of respiration rate for *C. gigas* larvae cover a range of larval sizes measured at 25°C (Gerdes 1983b) and 20°C (Hoegh-Guldberg & Manahan 1995) can be described by the relationship:

$$Resp = r_o W^n \tag{12}$$

where *Resp* is given in mL O_2 consumed ind^{-1} h^{-1} and W is animal dry tissue weight in mg. The base respiration rate, r_o, is assumed to reflect genetic variations in metabolic processes that are known to occur for individual *C. gigas* larvae (e.g., Lannan 1980). Hence, this parameter is specified using a distribution (described in a following section) that is assumed to represent metabolic variability within the larval population. Other coefficients are defined in Table 1. The respiration rates measured at 25°C (Fig. 7) were used along with the fractional changes in growth rate (Fig. 6) to obtain the full range of temperature and salinity effects on larval respiration. Respiration rate was converted to an energy demand using 20.21 J (mL O_2 consumed)$^{-1}$ to determine the metabolic cost of respiration.

Equation (12) provides the metabolic cost of respiration that must be met by the larva. As discussed in the previous section, the assimilated carbohydrate pool provides the first biochemical reservoir that is used to meet this demand (Table 2). This pool is converted to equivalent energy units using the conversions given previously, and the needed carbohydrate is removed from the pool. Any excess is added to the neutral lipid pool in an amount that is consistent with the carbohydrate-to-lipid ratio (Table 2).

If the assimilated carbon pool is insufficient to meet the cost of respiration, then the remaining deficit is taken from the neutral lipid pool and any remaining deficit is then taken proportionately from the structural components of the larva (Table 2). Periods during which the larva resorts to using structural material to cover metabolic costs result in reduction of larval condition index defined in the model as a reduction in the protein-to-ash ratio.

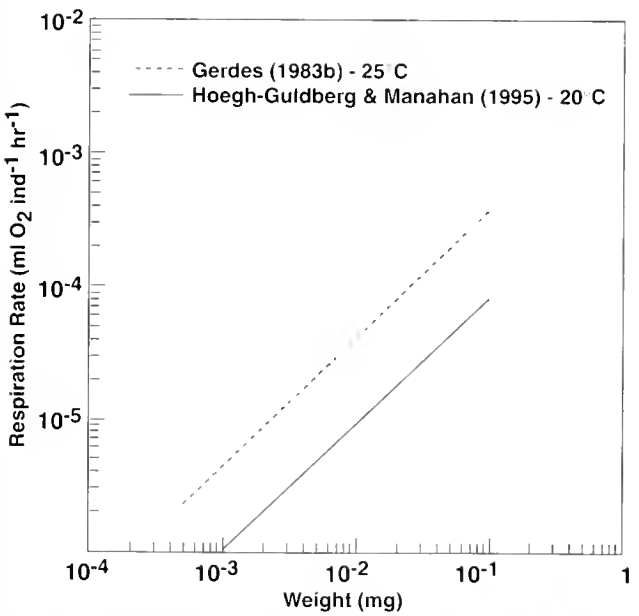

Figure 7. ***Crassostrea gigas*** **larval respiration rate measured as a function of larval weight at two temperatures.**

Larval Growth

Larval growth in a given time interval is based on maintaining the protein-to-ash ratio (Fig. 4, Eq. 5) for a given larval length. Larval growth resulting in an increase in length is assumed to occur when the protein, carbohydrate, and polar lipid pools are in excess of what is needed to maintain the protein-to-ash ratio at a given size. This is the excess net production (*ENP*) that determines α in Eq. 1.

Excess protein is obtained by subtracting from the protein pool the amount that is needed to maintain the ash weight at a given larval length, as is determined by the protein-to-ash ratio. The excess polar lipid and carbohydrate pools are computed from the excess protein pool based on the required structural ratios of these constituents. The excess net production for a given time interval is the sum of the excess protein, polar lipid, and carbohydrate. This gives the excess net production in a given time interval in terms of an increment in larval weight. The weight increment is then used with the length-to-dry tissue weight relationship (Fig. 2) to obtain an incremental increase in length for the increase in weight.

During times of protein deficit with respect to ash weight (low condition index), the larva can have a positive net production that increases organic mass and condition index, but produces no excess net production and, hence, no increase in length.

Larval Metamorphosis

Observations suggest that once *C. gigas* larvae reach 275 μm they may initiate metamorphosis and this process may or may not be successful (Ventilla 1984, Kusaki 1991, Laing 1995). Thus, in the model, the larva is assumed to have the potential of becoming competent for metamorphosis at 275 μm. Just prior to this point, at a length of 250 μm, filtration rate declines. Observations show that *C. gigas* larvae can become competent for metamorphosis over a range of sizes. This implies that the switch between Eqs. 6 and 7 controlling the point where filtration rate changes might contain a size dependency, determined by some metabolic process that is, as yet, unknown. Not having this information, the point at which the larva can become competent for metamorphosis was fixed at a size a little larger than the size observed by Gerdes (1983a) for the change in filtration rate. Simulations discussed subsequently support this decision.

Once the larva reaches 275 μm, it becomes competent to metamorphose if it experiences a 25% drop in neutral lipid stores in one day. This is determined by the interrelationship of food supply, filtration rate, and respiration rate. Competency is triggered by a decrease in neutral lipid that, if continued, would impair successful metamorphosis. Once competent, the larva immediately attempts metamorphosis. Successful completion of metamorphosis occurs if the larval neutral lipid pool is greater than the polar lipid pool. This establishes a minimum storage requirement needed to sustain metamorphosis. If this condition is not satisfied, then metamorphosis is unsuccessful and the larva dies.

Biochemically Determined Metabolic Mortality

The simulated larval growth prior to metamorphosis is based on maintaining specific ratios between protein, polar lipid, carbohydrate, and ash weight. Small variations in these ratios are allowed, consistent with changes that occur in the larva as it grows (cf. Fig. 4). However, large changes are not permissible. The interdependencies of the biochemical ratios results in the protein-to-ash ratio being a good indicator of the biochemical state of the larva. If this ratio is reduced at any time to 70% or less of its needed value, then larval condition index is too low and the larva is assumed to die. This condition is termed starvation in the model.

During the initial stages of larval growth, about the first two days, the larva does not filter efficiently (Fig. 5), and hence food ingestion is not usually sufficient to cover metabolic costs. During this period, it is assumed that the larva survives by using its stored neutral lipid supply. However, if during this period the neutral lipid supply approaches zero, the larva is assumed to have reached its metabolic point of no return and dies. Also, inability of the larva to maintain its required protein-to-lipid and protein-to-carbohydrate ratios results in death.

Model Implementation

Initial *C. gigas* Egg Size, Including Genetic Variability

The eggs spawned by *C. gigas* adults have an average size of 50 μm (Quayle 1988, Arakawa 1990). However, using this as the initial condition for the model resulted in mismatches in the initial simulated and observed length-to-weight relationships, which are based on larval size. Thus, simple egg diameter is not the appropriate metric for use with the length-to-weight and other conversions. The discrepancy arises because of the mismatch in volume of a spherical egg and the more ellipsoidal-shaped larva. Therefore, it was necessary to convert initial egg diameter to an equivalent larval size. This was done using a diameter-to-length conversion factor of 1.096 (Arakawa 1990) that conserves volume in going from a spherical egg to an ellipsoidal-shaped larva. Thus, a 50 μm egg is equivalent to a 54.8 μm larva.

C. virginica egg size is observed to range between 30 and 80 μm (Gallager et al. 1986). More limited information is available for *C. gigas*, but a similar range of egg sizes can be inferred (Breese & Malouf 1975). This variation was assumed to represent genetically or environmentally determined variability in the spawning population. Therefore, for each simulation, the initial conditions included a range of egg sizes.

To establish the initial biochemical composition of the egg, the larval size immediately post-hatch was used with the length-to-dry tissue weight relationship (Fig. 2) to calculate an initial dry weight, which in turn was used to obtain an initial ash weight value (Fig. 3). The protein component of the egg was then determined by multiplying the ash weight by the protein-to-ash ratio. The egg polar lipid content was determined by multiplying the protein content by the polar lipid-to-protein ratio. The carbohydrate content was taken to be 1% of the dry weight of the modified egg. Egg neutral lipid content was obtained by difference through subtracting the protein, polar lipid, and carbohydrate weight from the initial dry weight value. If this calculation resulted in a negative value of neutral lipid, which can occur for very small eggs, the egg was assumed to be nonviable.

Predation and Other Nonmetabolic Sources of Larval Mortality

The larval model provides, as output variables, the total time for larval development, larval size at the end of the simulation, and a description of why the simulation ended. Termination of a simulation occurs because of successful metamorphosis, unsuccessful metamorphosis, inappropriate metabolic ratios, and starvation. The simulated larval results are then examined with a submodel that calculates larval survivorship based on the timing of mortality and the larval life span of the survivors for each combination of egg size and respiration rate represented by the genetic variability assigned to the cohort. Losses to nonmetabolic sources of mortality, such as predation, are evaluated at this point with losses increasing in proportion to the larval life span obtained for each combination of egg size and respiration rate. The resultant simulated distributions of survivorship can then be compared with similar values reported from field and laboratory studies.

Predation and other forms of nonmetabolic mortality (*EM*) are imposed during the larval period using a relationship assumed to be of the form

$$EM(j,k) = e^{-m_{\nu}LD(j,k)} \tag{13}$$

where the daily mortality rate, m_{ν}, is the same as that used for *C. virginica* larvae (Dekshenieks et al. 1997) and *LD* is the total time required for a larva with an initial egg size (j) and respiration rate (k) to trigger a mortality event or to successfully metamorphose. The longer the larva takes to develop, the higher the chance of nonmetabolic mortality.

Genetic Effects on Larval Mortality

Growth, mortality, and other population processes are apportioned based on genetic variability, in which certain combinations of initial egg size and respiration rate are less common in the cohort and in which certain combinations are less viable overall,

either due to metabolic imbalances, metabolic inefficiencies, or longer larval life spans increasing nonmetabolic mortality. This type of genetically determined outcome, *GE*, is prescribed with a Gaussian function of the form:

$$GE = e^{-\left(\frac{ES - ES_o}{2sd_{ES}}\right)^2} - e^{-\left(\frac{Resp - Resp_o}{2sd_{Resp}}\right)^2} \quad (14)$$

where the Gaussian distributions extend for two standard deviations ($2sd_{egg}$, $2sd_{Resp}$) about a central egg size and respiration rate that are given by ES_o and $Resp_o$, respectively. Coefficient definitions and values are given in Table 1. Equation 14 weights mortality or any other population process by a population distribution that is characterized by a certain range of egg sizes and respiration rates. Thus, the surviving larval population represents the combined effects of genetics, food composition, and environmental conditions.

RESULTS

Reference Simulation

The reference simulation was run with near-optimal environmental conditions of 25°C, 30‰, a food concentration of 2 mg L^{-1}, and food with a protein, polar lipid, neutral lipid, carbohydrate ratio of 3:0.6:0.4:2.5. Development of *C. gigas* larvae over the first few days of larval life is primarily sustained by egg neutral lipid stores. The drawdown of neural lipid stores results in a decrease in the neutral lipid-to-protein ratio (Fig. 8A). The decrease in this ratio is most pronounced for eggs with small initial sizes. For initial egg sizes of 40 to 50 μm, the neutral lipid-to-protein ratio approaches zero. All larvae, independent of initial egg size, reach a maximum ratio value of between 0.15 and 0.165 and a size of ≥275 μm (Fig. 8B). Larvae arising from larger eggs reach these values earlier, and hence can metamorphose earlier. Once a size of 250 μm is reached, filtration rate declines and so, too, does the neutral lipid to protein ratio. However, growth continues and most larvae metamorphose at about 300 μm, regardless of initial egg size (Fig. 8B).

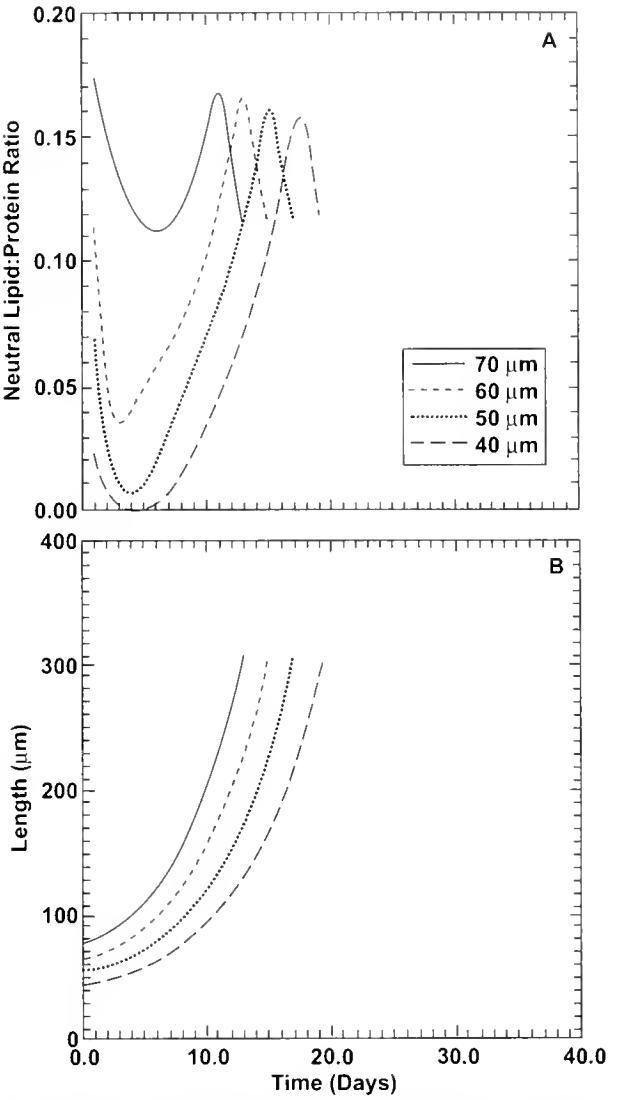

Figure 8. Simulated time development of the (A) neutral lipid-to-protein ratio and (B) *Crassostrea gigas* length obtained for a selection of initial egg sizes from 40 μm to 70 μm.

The simulated larval growth is exponential and independent of initial egg size (Fig. 8B). Larvae reach 100 μm, which corresponds to the small umbo stage (cf. Fig. 1), in 4 to 12 days for initial egg sizes of 70 μm and 40 μm, respectively. The rate of growth accelerates after 100 μm. The time required for the larvae to reach 300 μm ranges from 13 to 19 days for the largest and smallest initial egg sizes, respectively. The development time required for the 50 μm egg to reach 300 μm is 17 days, which agrees with development rates measured at 25°C and 30‰ (His et al. 1989). The range of simulated development times is also consistent with those reported for *C. gigas* larvae (Quayle 1988, Arakawa 1990, Laing 1995).

The effect of variations in initial egg size and growth efficiency on the fate of the larvae is summarized by the state of the larva at the time it either dies or successfully completes metamorphosis (Fig. 9). Variations in growth efficiency are modeled as variations in respiration rate; however, similar results would be obtained if the variation was in any component of Eq. 2. Initial egg sizes less than 37 μm result in nonviable larvae for all respiration rates. Initial egg sizes above 73 μm result in eggs that do not have sufficient neutral lipid stores after day 2 to continue development at all respiration rates. The large initial size of these eggs results in an imbalance in neutral lipids that cannot be corrected subsequently (cf. Fig. 8A). A similar fate occurs for initial egg sizes between 40 and 50 μm that yield larvae with high base respiration rates. For these larvae, too much of the neutral lipid pool is required to cover respiratory demand, and this produces a metabolic imbalance from which the larva cannot recover. Above and below the region of initial egg sizes and base respiration rates that produce successful metamorphosis are regions where the larva develops to the point of attempting metamorphosis, but is unable to do so successfully. A drop in neutral lipid triggers metamorphosis in these larvae, but they have insufficient lipid stores to cover the metabolic costs of metamorphosis.

At the population level, the fraction of the larvae that survive to complete metamorphosis is dependent on the initial egg size (Fig. 10A), with the distribution of survivorship centered around an initial egg weight of 50 μm, which is the center of the egg size distribution. Survivorship tapers off toward larger egg sizes, with essentially no survival to metamorphosis at egg sizes greater than 68 μm. Survivorship at smaller egg sizes decreases abruptly with essentially no survival at sizes less than 40 μm. Larval survivorship as a function of base respiration rate (Fig. 10B) is maximal at

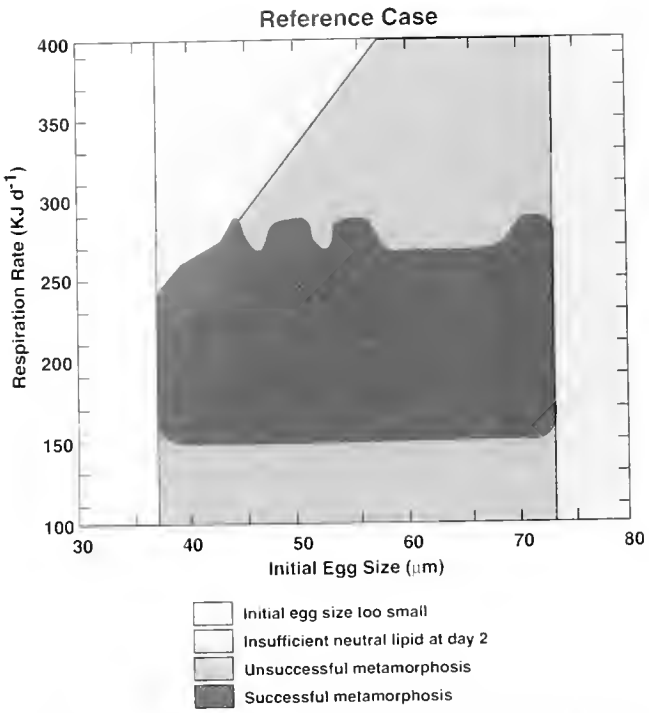

Figure 9. Simulated time development of *Crassostrea gigas* larvae for a range of initial egg sizes and base respiration rates. The simulation used the reference case environmental conditions of 25 C, 30‰, and a food concentration of 2 mg L^{-1} with a protein, polar lipid, neutral lipid, and carbohydrate ratio of 3:0.6:0.4:2.5, respectively.

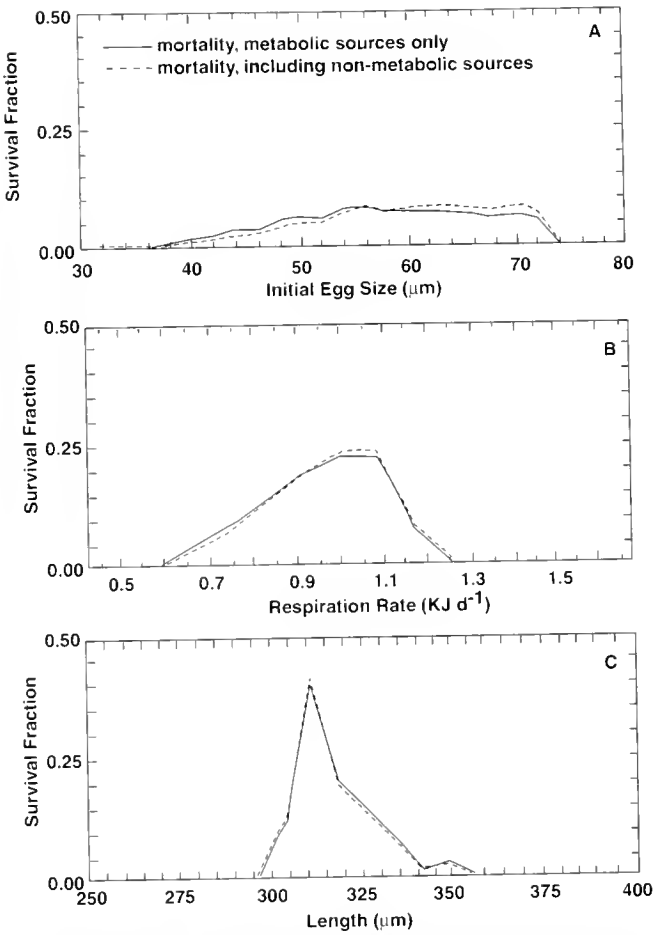

Figure 10. Simulated *Crassostrea gigas* larval survival as a function of (A) initial egg size, (B) base respiration rate, and (C) larval length at metamorphosis, using the reference case environmental conditions given in Figure 9.

rates around 1.05 KJ day^{-1}. Respiration rates above this result in an abrupt decrease in survival, with no survival at rates above 1.254 KJ day^{-1}. Larval survival at respiration rates below 1.05 KJ day^{-1} slowly decreases and is zero at rates below 0.628 KJ day^{-1}. Most larvae reach sizes between 300 and 325 µm before metamorphosis (Fig. 10C).

Detailed verification of the reference simulation is difficult because data on larval biochemical composition, as it varies with egg size, development time, and environmental conditions, are meager. General trends in larval success as measured by survivorship, larval size, and larval life span are much better known. Four such trends are observed in the simulation. (1) Adequate neutral lipid stores are a prerequisite of high survival during the critical period a few days post-hatch and at metamorphosis. The reference simulation demonstrates both effects (Figs. 8A, 10B). (2) Typically, egg size ranges from 40–60 µm. The reference simulation identifies this range as optimal for *C. gigas*, based on changes in biochemical composition dictated by larval energetics (Fig. 10A). (3) Successful metamorphosis occurs for larvae of 295–340 µm in the reference simulation. This is a frequently observed size range (Ventilla 1984, Kusaki 1991, Laing 1995). (4) Larval life span for the most successful egg sizes varies from about 14 to 17 days, a range that approximates the norm in observation (Ventilla 1984, Arakawa 1990, Laing 1995).

Effect of Larval Filtration

The parameterization for larval filtration (Eqs. 6 and 7, Fig. 5) is based on the assumption that the filtration response changes abruptly at 250 µm. *C. gigas* larvae are observed to metamorphose at a range of lengths. The change in filtration rate is an important contributor to the mechanisms by which the model determines the onset and success of metamorphosis. Therefore, the larval length at which the change in filtration response occurs was varied.

Varying the onset of a decline in filtration rate does not impact the range of viable egg sizes because this range is determined by events that predate this point in the larva's life span (Figs. 11A, 11B). However, if filtration rate changes at 270 µm, larvae spend a longer time at a high filtration rate. These animals metamorphose successfully over a wider range of base respiration rates than those that support successful metamorphosis when filtration rate begins to decline at 250 µm (Fig. 12B). Also, the overall population survivorship is higher; 78.8% of the cohort versus 62.4% in the reference simulation (Figs. 9, 10), and the range of lengths at which larvae metamorphose is wider (Fig. 12C).

Moving the larval length at which the filtration rate response changes to a smaller size, 230 µm, results in a limited range of base respiration rates at which successful metamorphosis can occur (Figs. 11B, 13B). Many of the animals survive to attempt metamorphosis, but are unsuccessful at completing the process. The smaller size at which the filtration rate is reduced results in the larvae not storing enough neutral lipid to cover the metabolic needs associated with metamorphosis. Larval length at metamorphosis (Fig. 13C) is now limited to a small range of sizes.

Total population survivorship drops from 62.4% in the refer-

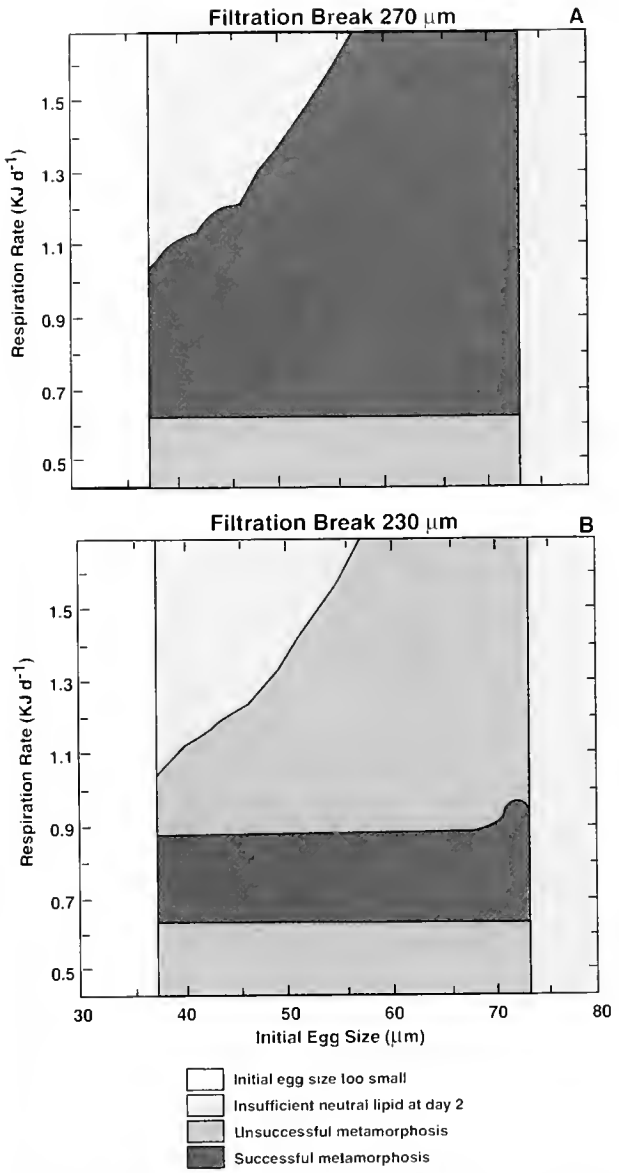

Figure 11. Simulated fate of *Crassostrea gigas* larvae for a range of initial egg sizes and base respiration rates in which the change in larval filtration rate response is at (A) 270 µm and (B) 230 µm. The simulation used the reference case environmental conditions given in Figure 9.

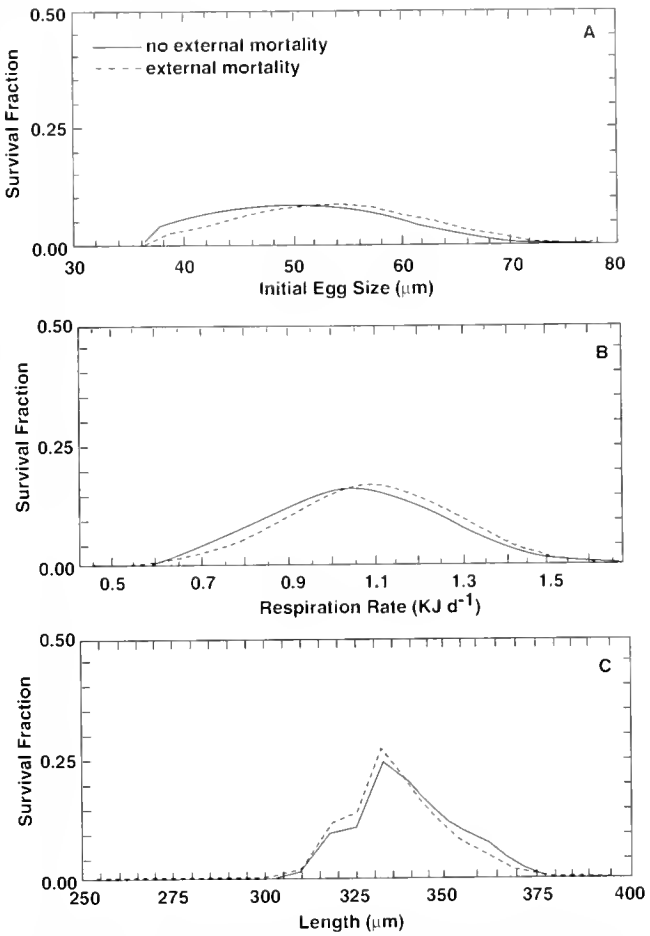

Figure 12. Simulated *Crassostrea gigas* larval survival as a function of (A) initial egg size, (B) base respiration rate, and (C) larval length at metamorphosis obtained when the change in larval filtration rate response occurs at 270 µm. The simulation used the reference case environmental conditions given in Figure 9.

ence case to 8.1% with a size trigger of 210 µm rather than 250 µm. Cohort survivorship declines dramatically at trigger sizes below 250 µm (Table 3) and asymptotes rapidly above 250 µm. At trigger sizes above 250 µm, some larvae do not metamorphose until reaching sizes much larger than normally observed (e.g., 375–400 µm, Fig. 12C), however. Accordingly, larval growth, as modeled, requires a filtration rate trigger near 250 µm to obtain observed levels of cohort survivorship and size at metamorphosis. This approximates the size where filtration rate declines are observed to take place (Gerdes 1983a).

Effect of Diet

Information on the effects of diet on growth and survival of *C. gigas* larvae indicate that high-lipid and low-protein diets are usually efficacious (Utting 1986, Thompson & Harrison 1992, Thompson et al. 1994, 1996). These trends are reproduced by the model.

A larval diet that is lacking in neutral lipid but contains the correct ratios of protein, polar lipid, and carbohydrate (3:0.6:0:2.5) results in animals that are unable to successfully complete metamorphosis at all ranges of initial egg size and base respiration rate (Fig. 14). Lack of neutral lipids results in more of the protein and carbohydrate pools being used to cover the demands of respiration and growth and, so, lipid stores are insufficient to sustain metamorphosis. In addition, the range of egg sizes and base respiration rates in which the neutral lipid store at day 2 is insufficient for further development is greatly expanded relative to what is obtained for a diet containing neutral lipid (Fig. 14 versus Fig. 9).

A diet in which the protein content is 50% higher relative to the standard diet (4:0.6:0.4:2.5) produces a similar result in that no combination of initial egg size or respiration rate results in successful metamorphosis (Fig. 15A). Larvae either have insufficient neutral lipid reserves at day 2 to continue development or attempt metamorphosis but fail to complete the process. A high-protein diet requires that more of the neutral lipid stores be used to cover tissue structural needs, and hence less lipid is stored for later use in metamorphosis.

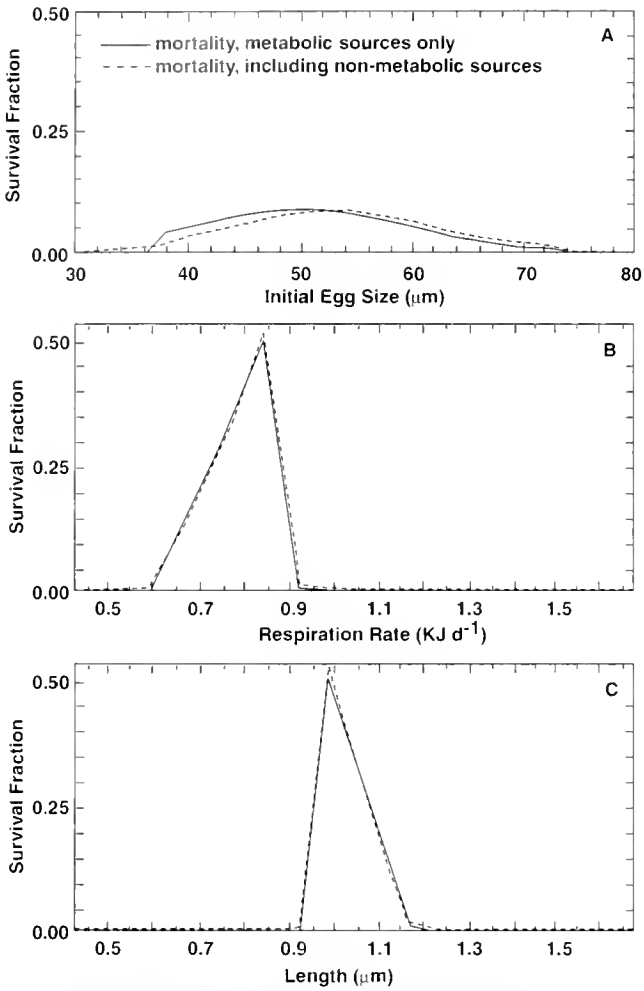

Figure 13. Simulated *Crassostrea gigas* larval survival as a function of (A) initial egg size, (B) base respiration rate, and (C) larval length at metamorphosis obtained when the change in larval filtration rate response occurs at 230 μm. The simulation used the reference case environmental conditions given in Figure 9.

A diet low in protein (2:0.6:0.4:2.5) is beneficial to the larva (Fig. 15B). Ingestion of low-protein food extends the range of respiration rates that result in successful metamorphosis (Fig. 15B versus Fig. 9). More polar lipid and carbohydrate is available to cover metabolic costs and to increase neutral lipid stores. This increases metamorphosis success. Simulations indicate that a low-protein diet increases overall population survivorship under hatchery conditions where nonmetabolic causes of mortality are minimized (88.8% from 62.4% in the reference simulation), but decreases survivorship under field conditions, from 6.1% in the reference simulation to 4.7%, due to slower growth and longer planktonic times increasing losses to predation.

TABLE 3.

Total population survivorship for simulated cohorts of *Crassostrea gigas* larvae in which the larval size triggering a change in filtration rate was varied from 210 μm to 290 μm.

Trigger size	210	230	250	270	290
Cohort survivorship	8.1%	17.2%	62.4%	78.8%	78.8%

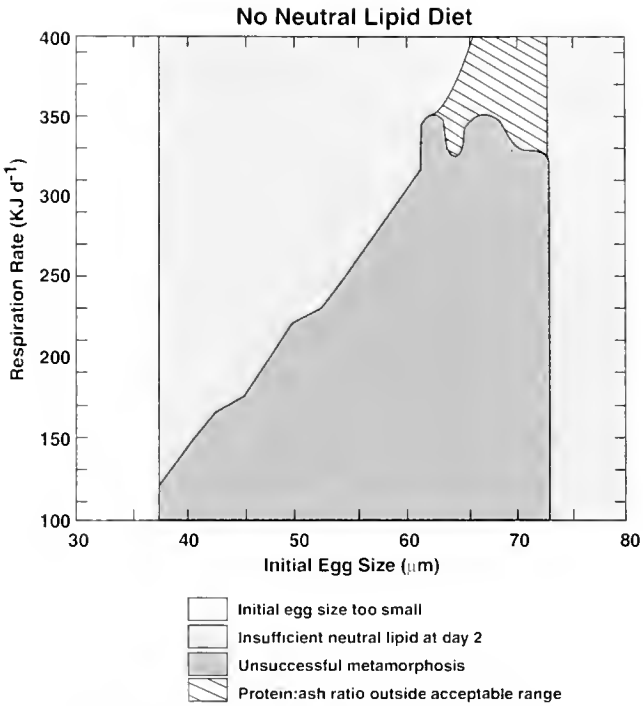

Figure 14. Simulated fate of *Crassostrea gigas* larvae for a range of initial egg sizes and respiration rates resulting when the larval diet includes no neutral lipid. The simulation used the reference case environmental conditions given in Figure 9, except that the food had a protein, polar lipid, neutral lipid, and carbohydrate ratio of 3:0.6:0:2.5.

Effect on Metamorphosis

The cue that is assumed to initiate metamorphosis is a 25% drop in the neutral lipid reserves of the larva over a span of one day. This level of decline was chosen as a metamorphosis trigger by comparing the simulated size and biochemical composition of metamorphosing larvae to measured values. The condition and size of larvae at metamorphosis is quite variable, and the metamorphosis trigger value used in the model represents one that simulates the average of these conditions.

Requiring only a 10% drop in neutral lipids in one day to trigger metamorphosis results in essentially all combinations of initial egg size and base respiration rate producing larvae that successfully undergo metamorphosis (Fig. 16 versus Fig. 9). The lower trigger value allows larvae to have a greater energy store at the time metamorphosis is attempted, and hence the probability of success is increased. At the population level, a wide range of egg sizes and base respiration rates results in survival (Figs. 17A & 17B), but larval length at metamorphosis is shifted to smaller larvae (Fig. 17C). The population mode in this case extends from 285 μm to about 305 μm, a size range predominately comprising sizes smaller than typically observed and smaller than the 295 μm to about 335 μm size range observed in the reference simulation (Fig. 9C). Increasing the trigger to a 40% reduction in neutral lipids in one day (not shown) results in attempted, but failed, metamorphosis at all initial egg sizes and base respiration rates. Thus, the value of 25% produces observed success rates for metamorphosis at a range of observed egg sizes not achieved by higher or lower trigger values.

Metamorphosis success is determined by the ratio of neutral

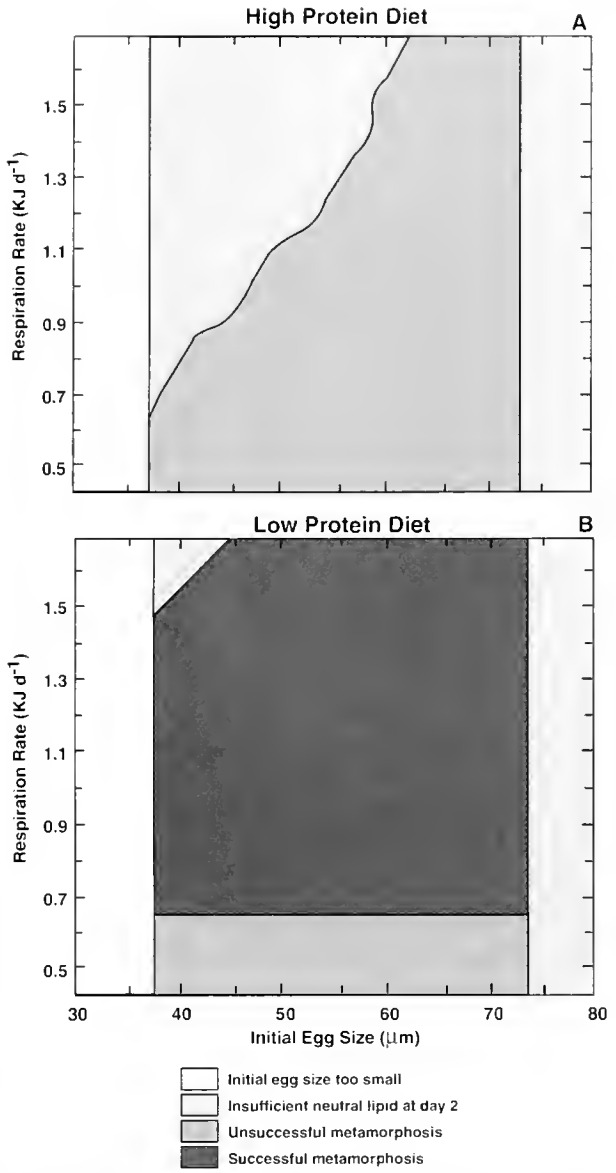

Figure 15. Simulated fate of *Crassostrea gigas* larvae for a range of initial egg sizes and respiration rates resulting when the larval diet is (A) high in protein and (B) low in protein. The simulation used the reference case environmental conditions given in Figure 9, except that the food had a protein, polar lipid, neutral lipid, and carbohydrate ratios of 4:0.6:0.4:2.5 and 2.5:0.6:0.4:2.5.

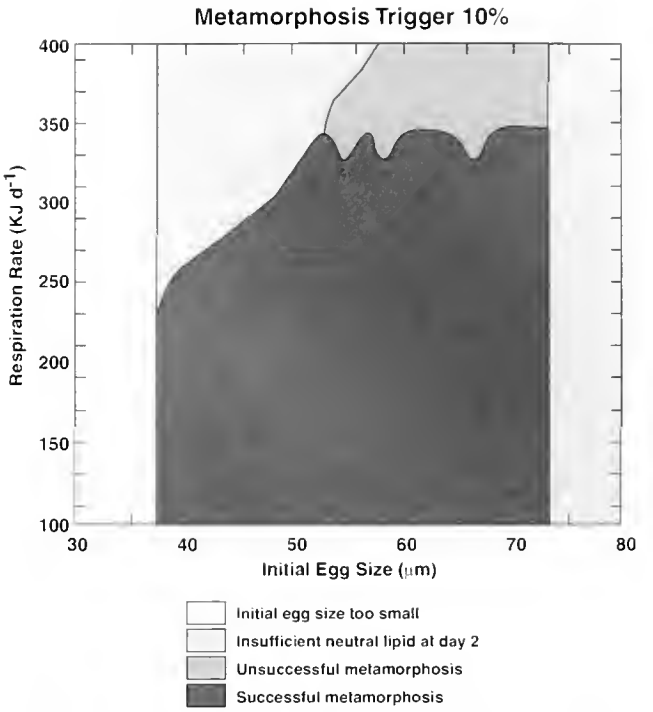

Figure 16. Simulated fate of *Crassostrea gigas* larvae for a range of initial egg sizes and base respiration rates when the trigger for metamorphosis is set to be a 10% drop in neutral lipid stores in one day. The simulation used the reference case environmental conditions given in Figure 9.

lipid to polar lipid in the animal at the time that metamorphosis is triggered. In the reference case (Fig. 9), successful metamorphosis occurs if the content of neutral lipid exceeds the content of polar lipid. Allowing metamorphosis to be successful when the larval neutral lipid reserves are greater than 80% of the polar lipid content produces successful metamorphosis over a large range of initial egg sizes and respiration rates (Fig. 18A). At the population level, larval survival is enhanced over a wide range of initial egg sizes and base respiration rates (Fig. 19A, 19B). The length at metamorphosis also extends over a wide range (Fig. 19C), wider than normally observed, but the mode of the population is still around 308 µm, as seen in the reference simulation (cf. Fig. 10C).

If metamorphosis is successful only when the neutral lipid pool is greater than 110% of the polar lipid pool, the range of initial egg sizes and respiration rates at which successful metamorphosis occurs is greatly reduced (Fig. 18B). The effect of egg size on larval survivorship is only somewhat modified (Fig. 20A); however, the range of base respiration rates that result in successful metamorphosis is greatly reduced (Fig. 20B), as is the range of larval lengths (Fig. 20C). Overall, trigger values above 100%, a 1:1 neutral lipid-to-polar lipid ratio, generate rates of successful metamorphosis that are too low and larval sizes at metamorphosis that are too large (Table 4). Trigger values below 100% at first seem to be defensible; however, survivorships are unusually high. Hence, model conditions chosen for the reference simulation include, as the minimally required neutral lipid stores for success at metamorphosis, a quantity greater than the polar lipid content of the larva (>1:1). Recall that the polar lipid content is constrained to a predetermined ratio with protein and structural carbohydrate, and so the same results would have occurred had neutral lipid been compared to any structural constituent.

Temperature and Salinity Effects

The temperature and salinity used for the previous simulations, 25° C and 30‰, is near optimal for the growth and development of *C. gigas* larvae (cf. Fig. 6). The optimal range of these environmental variables is narrow, and therefore relatively small changes in these conditions have the potential of causing large changes in larval growth and development. Population survivorship is nonzero over a relatively narrow temperature range and a somewhat wider salinity range (Table 5). A 5°C reduction in temperature, from 25°C to 20°C for example, results in no combination of initial egg size or base respiration rate that produces successful metamorphosis (Fig. 21A). A reduction in salinity to 20‰ extends the

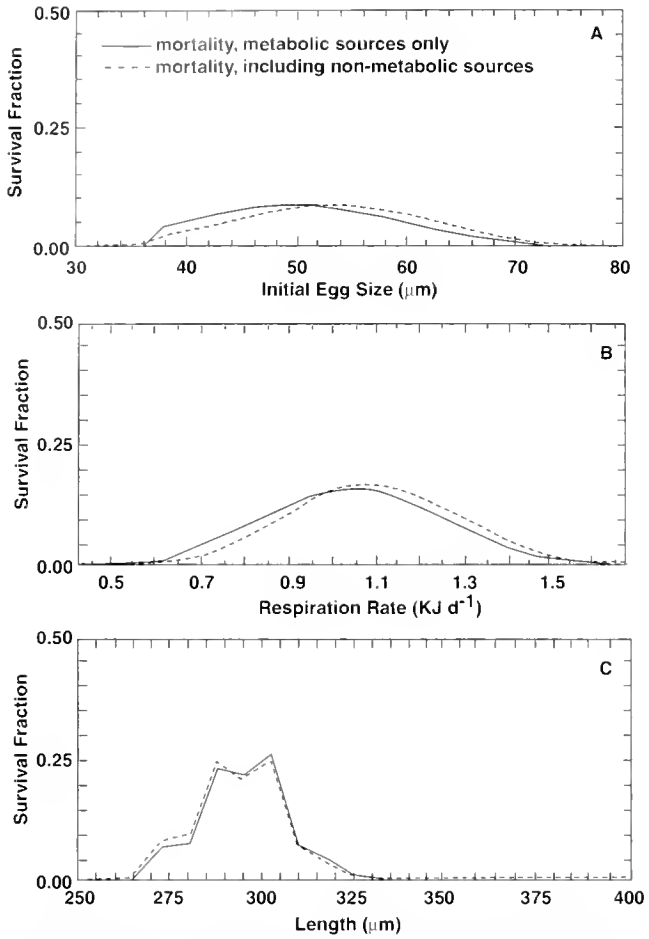

Figure 17. Simulated *Crassostrea gigas* larval survival as a function of (A) initial egg size, (B) respiration rate, and (C) larval length at metamorphosis obtained when metamorphosis occurs in response to a 10% drop in neutral lipid stores in one day. The simulation used the reference case environmental conditions given in Figure 9.

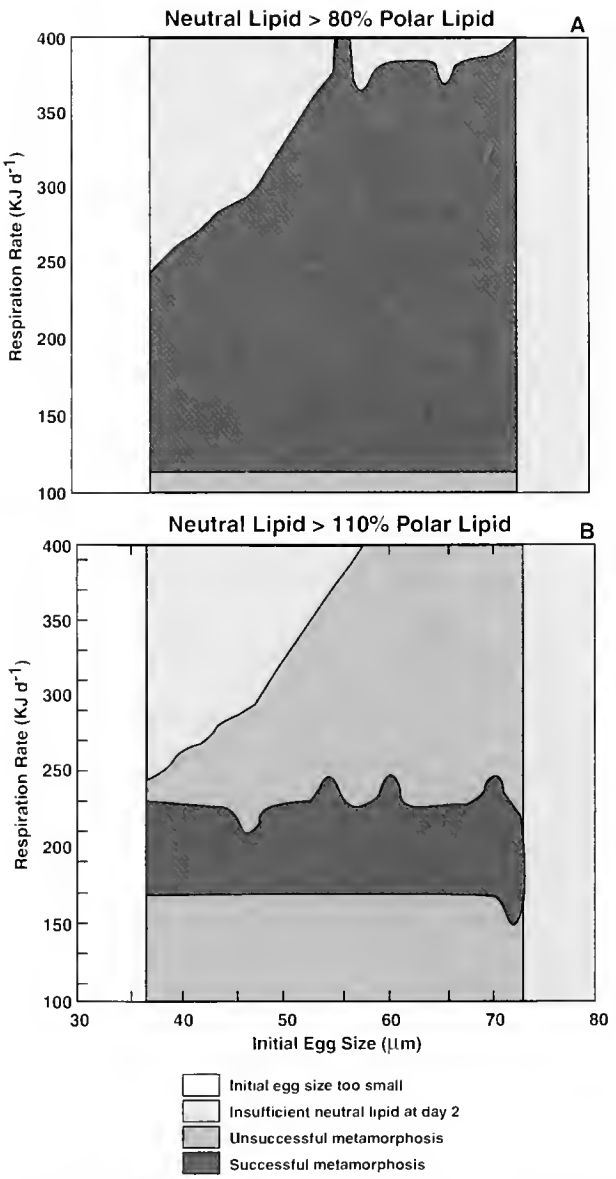

Figure 18. Simulated fate of *Crassostrea gigas* larvae for a range of initial egg sizes and respiration rates when the metamorphosis trigger occurs when (A) neutral lipids are greater than 80% of the polar lipid stores and (B) neutral lipids are greater than 110% of the polar lipid stores. The simulation used the reference case environmental conditions given in Figure 9.

range of initial egg weights and base respiration rates at which the larva exceeds its neutral lipid constraint at day 2 of development (Fig. 21B). Half as many animals exposed to this salinity successfully complete metamorphosis as in the reference case.

Effect of Variations in Food Resources

Environmental food concentration can vary over a wide range. Food concentration in excess of 2 mg L^{-1} used for the reference simulation (cf. Fig. 9) only extends somewhat the range of initial egg weights and base respiration rates that result in successful metamorphosis because 2 mg L^{-1} food is a saturating food concentration. However, a 50% reduction in food concentration, from 2 mg L^{-1} to 1 mg L^{-1}, significantly narrows the range of base respiration rates that result in successful metamorphosis (Fig. 22A). The strong coupling between respiration rate and food availability is not surprising since respiration is the primary metabolic loss that the larva must cover through ingestion. This result is further substantiated when looking at population level survival trends (Table 6). Survivorship declines rapidly at food concentrations below 2 mg L^{-1} and reaches zero at food concentrations of about 0.5 mg L^{-1}.

Certain environmental conditions may spare a decrease in food supply by permitting an increase in filtration rate. Increasing temperature in the previous simulation to 30°C, for example, increases survival by permitting some larvae with high respiratory demands to survive (Fig. 22B). Total population survival increases from 27.2% to 37.6% at the higher temperature.

Planktonic values of food of 0.5 mg L^{-1} or less are not unusual. Consequently, larvae may experience times of starvation due to significantly reduced food concentrations. A simulation in which food is available at a concentration of 2 mg L^{-1} for days 1 to 4 of larval development and unavailable after day 5 shows that no combination of initial egg size and base respiration rate results in successful metamorphosis (Fig. 23A). Death results from poor condition in some cases, but more frequently from metabolic imbalances between principal biochemical constituents. Simulated

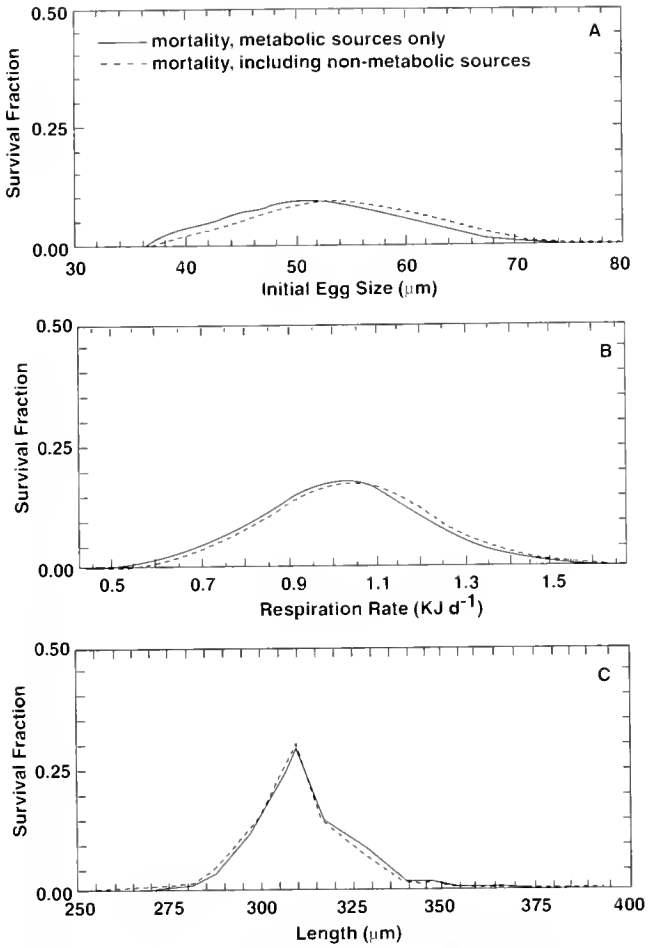

Figure 19. Simulated *Crassostrea gigas* larval survival as a function of (A) initial egg size, (B) base respiration rate, and (C) larval length at metamorphosis obtained when metamorphosis occurs when neutral lipids are greater than 80% of polar lipid. The simulation used the reference case environmental conditions given in Figure 9.

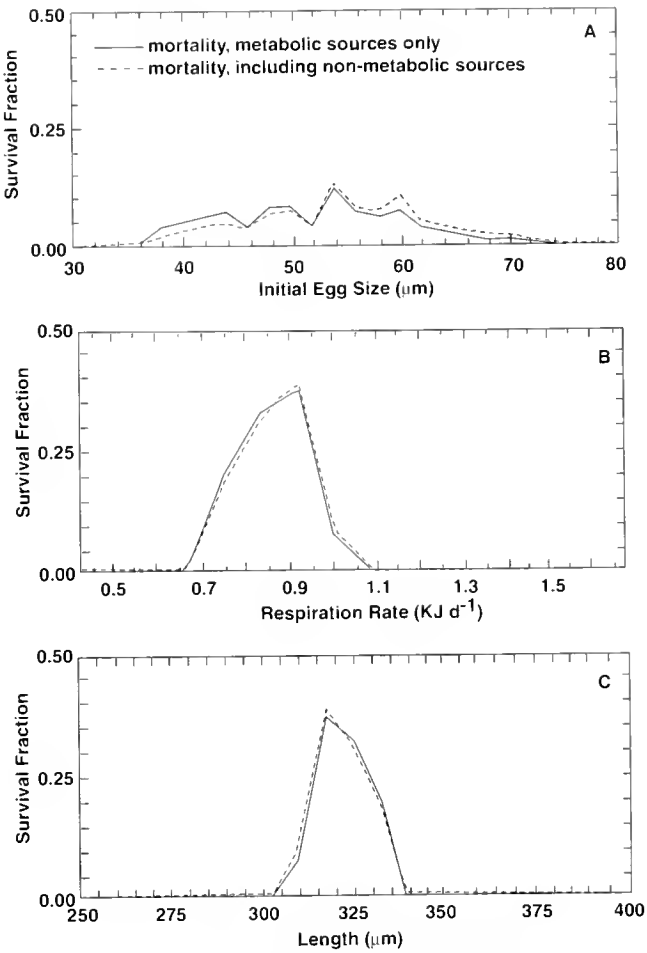

Figure 20. Simulated *Crassostrea gigas* larval survival as a function of (A) initial egg-size, (B) base respiration rate, and (C) larval length at metamorphosis obtained when metamorphosis occurs when neutral lipids are greater than 110% of polar lipid. The simulation used the reference case environmental conditions given in Figure 9.

survival times were similar to those observed for starved larvae (His & Seaman 1992, Laing 1995). Survival times of simulated larvae starved after day 4 were two to eight days, with most dying within two days. Larvae starved after day 9 survived two to ten days, with most dying in the first four days. Thirty-three percent of larvae starved after day 14 completed metamorphosis. These were larvae that came from relatively large eggs with low base respiration rates (Fig. 23B). Such larvae were much closer to metamorphosis on day 14 when starvation began than other larvae and were able to complete metamorphosis using their energy stores.

This last simulation is particularly interesting because a narrower range in egg size produced successful larvae. In other simulations, varying base respiration rate was much more significant in determining survival than varying egg size. The simulation supports the conclusion of Gallager et al. (1986) and Gallager and Mann (1986b) that egg quality is important in minimizing losses due to low food supply during larval life.

Genetics of Egg Size and Respiration Rate

As discussed previously, it was necessary to model larval cohorts characterized by a range of egg sizes and growth efficiencies to obtain the observed ranges in larval life span, size at metamorphosis, and survivorship seen in experimental studies of *C. gigas* larvae. One important choice, then, was the mean of the frequency distribution chosen for these two variable characters.

The average egg size was taken to be 50 μm in the reference simulation. With a few exceptions, varying the conditions of the simulation did not vary the range of viable egg sizes to a great degree (Figs. 9–22). The range of viable egg sizes is dictated by the most basic constraints imposed by biochemical composition at birth and by environment (e.g., food supply) rather than by genetics. Not surprisingly, changing the initial egg distribution so that it is centered on a 60 μm egg, rather than a 50 μm egg, results in survivorship that is skewed toward larger egg sizes (Fig. 24A versus 9A) because most eggs of 40 to 70 μm in size are viable

TABLE 4.

Total population survivorship for simulated cohorts of *Crassostrea gigas* larvae in which successful metamorphosis occurs when neutral lipid reserves exceed by a given fraction the polar lipid content.

Neutral lipid-to-polar lipid ratio	0.8:1	1.0:1	1.1:1	1.2:1
Cohort survivorship	80.5%	62.4%	26.5%	0.0%

TABLE 5.

Total population survivorship for simulated cohorts of *Crassostrea gigas* larvae exposed continuously to different temperatures (° C) and salinities (‰).

Temperature	15	20	25	30	35
Cohort survivorship	0.0%	0.0%	62.4%	63.3%	0.0%
Salinity	15	20	25	30	35
Cohort survivorship	0.0%	33.1%	63.3%	62.4%	31.8%

(Fig. 9), and moving the average size higher simply increases the total number of eggs spawned in this higher size range. Respiratory effects on survivorship (Fig. 24B) are not altered by a change to mean egg size. However, the larval length at the time of metamorphosis is somewhat larger (Fig. 24C). Total survivorship declines somewhat from 64% to 60% because more of the cohort that is spawned falls into egg sizes above 70 µm. Overall, however, the simulation shows that a moderate change in mean egg size does not materially change the outcome of the simulation. As the simu-

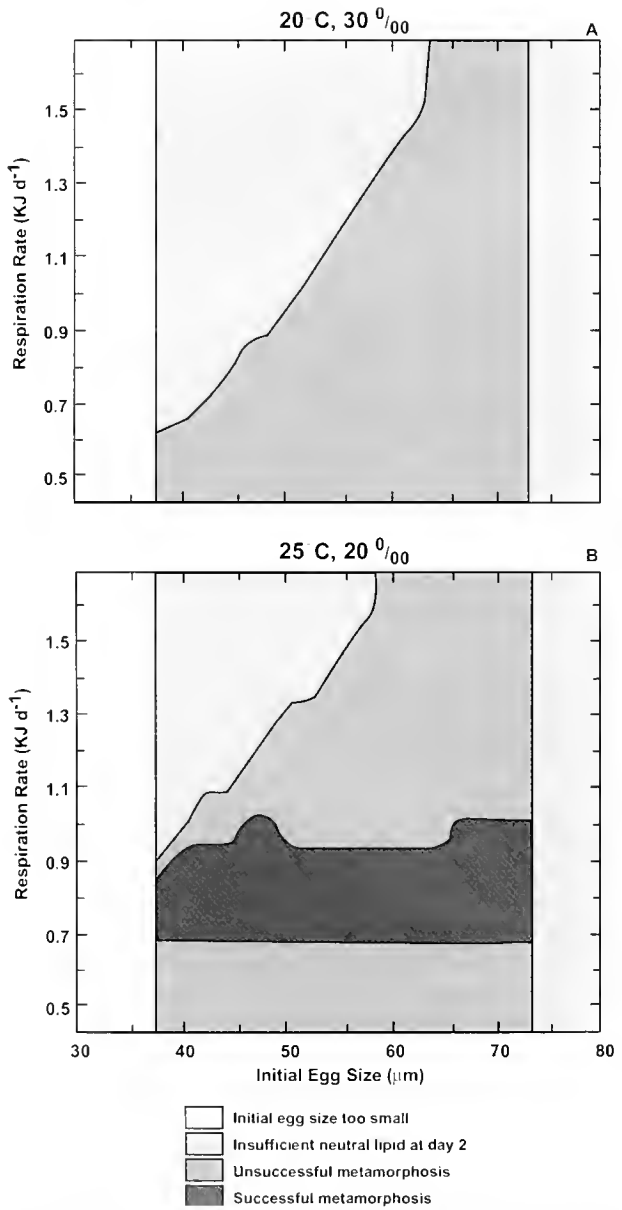

Figure 21. Simulated fate of *Crassostrea gigas* larvae for a range of initial egg sizes and base respiration rates at (A) 20 C, 30‰ and (B) 25 C, 20‰. Food concentration was 2 mg L^{-1} with a protein, polar lipid, neutral lipid, and carbohydrate ratio of 3:0.6:0.4:2.5.

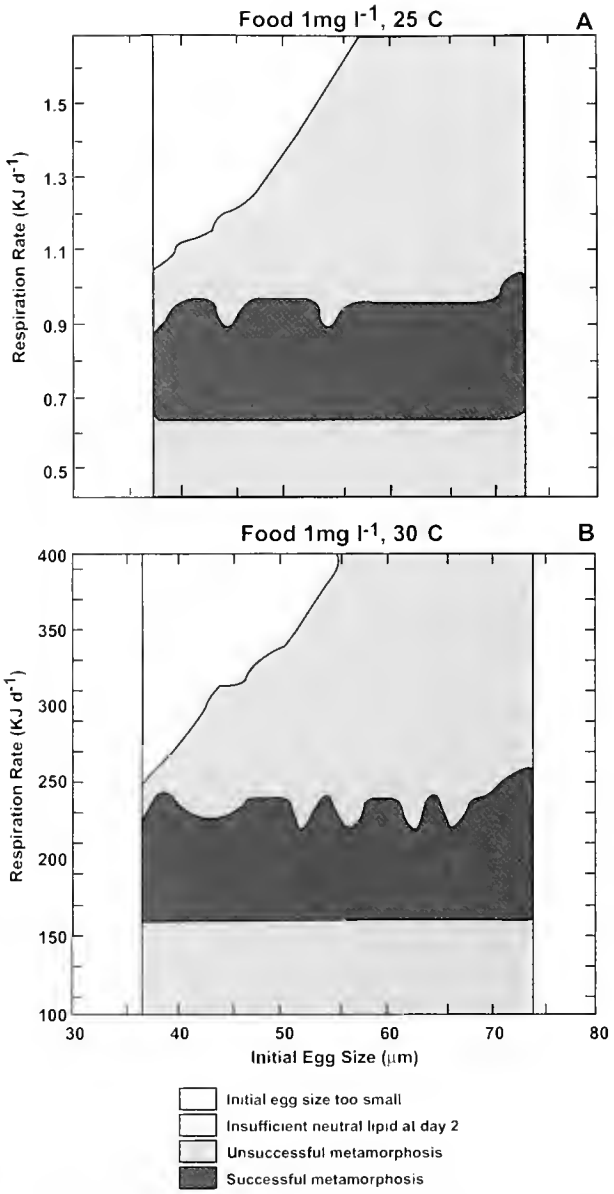

Figure 22. Simulated fate of *Crassostrea gigas* larvae for a range of initial egg sizes and base respiration rates and for a food concentration of 1 mg L^{-1} at (A) 25°C and (B) 30 C. The simulation used environmental conditions of 30‰ and a food composition with a protein, polar lipid, neutral lipid, and carbohydrate ratio of 3:0.6:0.4:2.5.

TABLE 6.

Total population survivorship for simulated cohorts of *Crassostrea gigas* larvae exposed continuously to different concentrations of food (mg L^{-1}).

Food	0.5	1.0	2.0	4.0
Cohort survivorship	0.0%	27.2%	62.4%	64.4%

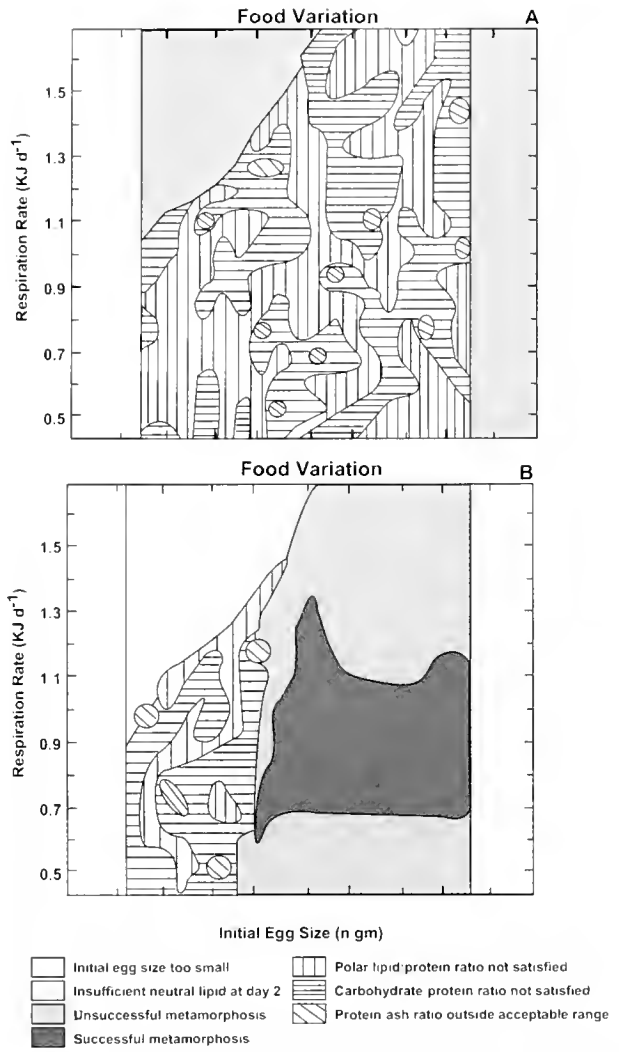

Figure 23. Simulated fate of *Crassostrea gigas* larvae for a range of initial egg sizes and base respiration rates and (A) a food concentration of 2 mg^{-1} available for days 1 to 4 and zero afterward and (B) a food concentration of 2 mg^{-1} available for days 1 to 14 and zero afterward. The simulation used environmental conditions of 25 C, 30‰ and a food composition with a protein, polar lipid, neutral lipid, and carbohydrate ratio of 3:0.6:0.4:2.5.

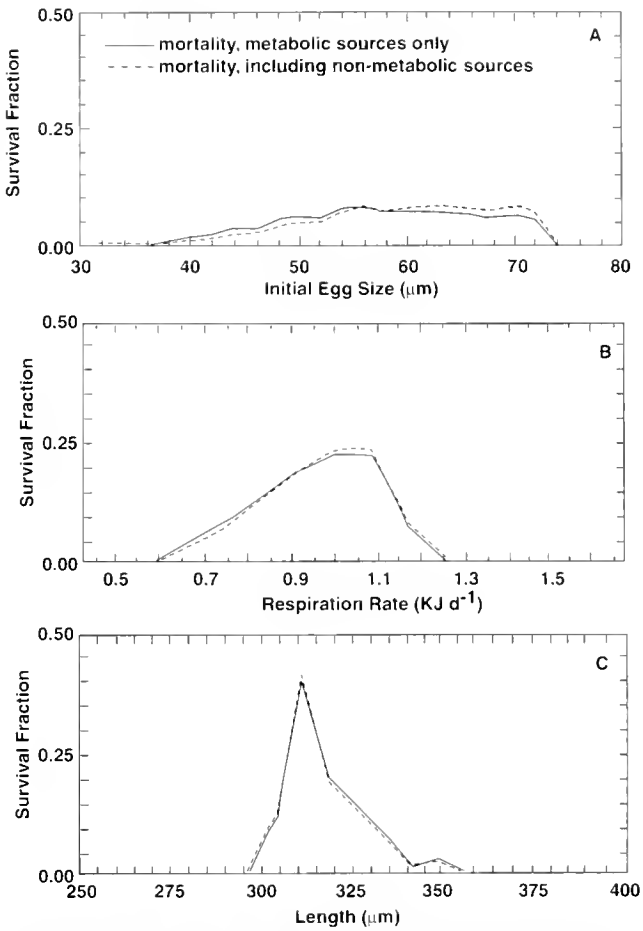

Figure 24. Simulated *Crassostrea gigas* larval survival as a function of (A) initial egg size, (B) base respiration rate, and (C) larval length at metamorphosis for an initial egg size distribution that is centered at 60 μm. The simulation used the reference case environmental conditions given in Figure 9.

lation depicted in Figure 23B shows, this outcome may not be repeated in cases of nonsaturating food supply.

Average base respiration rate for larvae was set at 1.05 KJ day^{-1}. Increasing the mean base respiration rate by 20% to 1.25 KJ day^{-1} results in larval survival skewed toward lower respiration rates (Fig. 25B) and smaller egg sizes (Fig. 25A), with a rapid decrease in survival above respiration rates of 1.05 KJ day^{-1} and egg sizes above 60 μm. Larval survival peaks at a metamorphosis length of 308 μm, which is similar to that obtained in the reference simulation (Fig. 9C). However, larval survivorship decreases rapidly for larger larvae (Fig. 25C). Total survivorship declines dramatically from 64% to 33%. Higher base respiration rates penalize larger larvae because insufficient excess neutral lipid can be obtained under the food supply provided to cover tissue maintenance and provide the requisite energy stores for metamorphosis. Thus, the model is considerably more sensitive to moderate changes in base respiration rate than in mean egg size. Conversely, the influence of environment on the range of viable base respiration rates is much greater than on the range of viable egg sizes and, so, a change in mean respiration rate might easily produce an alternate result under different environmental conditions.

DISCUSSION

Perspective

The model described here is unique in that it seeks to recreate many of the growth and mortality phenomena observed in *C. gigas* larvae from basic biochemical principals. The approach was dictated by a desire to model the influence of food quality and short-term changes in food supply on larval growth and survival and the influence of egg size and composition on ultimate success at metamorphosis.

Although biochemically based, the model contains only the crudest biochemical constructions. The larva is modeled as a four-constituent organism composed of protein, carbohydrate, neutral lipid, and polar lipid. Each constituent and the transitions between constituents are modeled using the simplest of flow schemes. So, for example, lipid and carbohydrate are interchangeable, carbohydrate covers respiratory demand when in sufficient supply, and

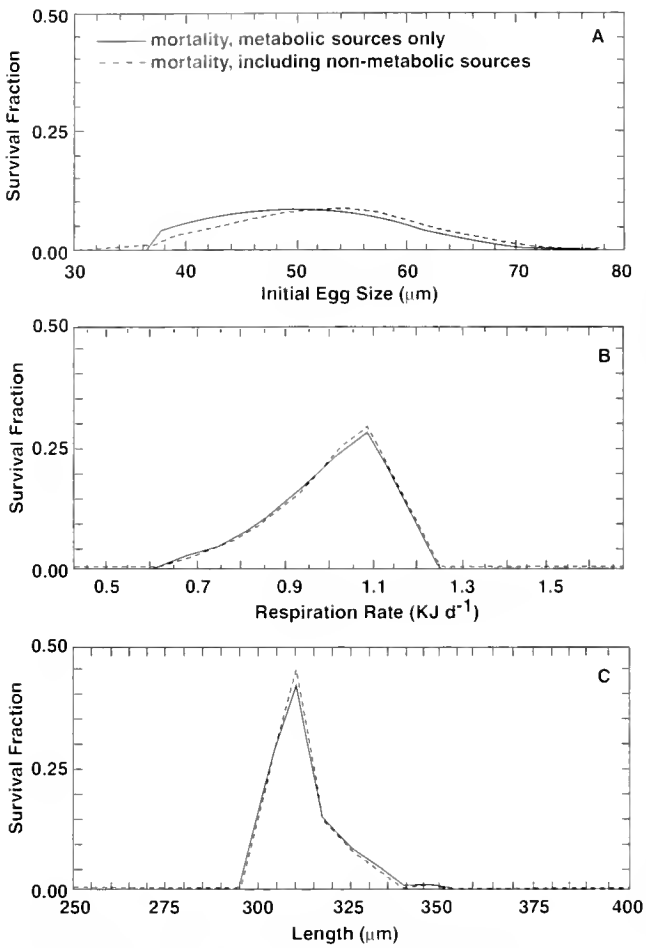

Figure 25. Simulated *Crassostrea gigas* larval survival as a function of (A) initial egg size, (B) base respiration rate, and (C) larval length at metamorphosis for a base respiration rate distribution that is centered at 1.254 KJ day^{-1}. The simulation used the reference case environmental conditions given in Figure 9.

neutral lipid is the primary storage component. Assimilated protein is used only to create tissue protein, and this necessitates the formation of a certain amount of structural carbohydrate and polar lipid. Failure to supply these other components in the amounts required by protein assimilation results in structural imbalances and eventually death. More complex biochemical transformations are excluded. So, for example, although many amino acids can be synthesized, protein, in the model, comes only from protein ingested as food, with the one exception early in larval life when neutral lipid is used to sustain growth in the first few days after birth. Carbohydrate and protein, though potentially used as energy reserves for metamorphosis or to sustain the larva during periods of negative scope for growth, are only used as the last resort and, then, only in proportion to their fractional contribution to structural tissues.

Although based on a simplistic biochemistry, the model succeeds in simulating some of the basic observations of *C. gigas* eggs and larvae, suggesting that simple biochemical constructs can be successful and may encompass the biochemical transitions most prominent in determining cohort success. Verifications of many of the details in the simulations cannot be accomplished because of limited information on the details of larval biochemical composition as it is influenced by environmental and genetic factors. Nevertheless, the model succeeds in simulating known population-level characteristics that permit verification at this higher-order level of integration.

Necessities of Model Construction

A number of special model constructs were required to obtain verifiable simulations. These included resolving a mismatch between egg ash content and earliest larva ash content, the conversion of egg size to earliest larval size, the addition of genetic variation, and the need to modify filtration rate, especially in early stage larvae.

Birth and Condition

Condition is tracked in the model using a protein-to-ash ratio. Tracking condition was necessitated by the desire to model periods of low food supply, including, in the extreme, periods of starvation. Particularly once the shell is formed, larval size does not change during periods of restricted food supply, but larval tissue weight does (Laing 1995).

The protein-to-ash ratio for eggs is high and does not fit the larval pattern. Hence, initializing the model with the protein-to-ash ratio of the egg consistently failed to produce verifiable simulations. Presumably, as the egg develops and hatches, ash is added from inorganic solutes in the surrounding water and the protein-to-ash ratio drops. The model was initialized with the protein-to-ash ratio of newly hatched larvae to circumvent this problem. Reconciling the mismatch between the protein-to-ash ratio of eggs and newly hatched larvae will require additional experimental studies.

Length at Birth

Eggs are more or less spherical. Larvae, even newly hatched, are not. The model tracks length independently of weight, a necessity imposed by the wealth of data for verification provided in terms of length and the need to follow condition. Initializing the model with egg "length" (diameter) fails because the increase in length during egg development is not representative of growth, but simply a result of tissue reorganization. Consequently, a growth model cannot account for this process. We used a conversion from egg diameter to earliest larval length to circumvent this problem.

Genetic Variation

One of the key observations recorded in the literature is the success rate at metamorphosis and the size of metamorphosing larvae. Considerable variability exists depending upon the conditions of larval culture, and egg quality provides a sufficiently strong signal that variations in egg quality should influence success at metamorphosis in simulated spawns. Considerable variability also exists within cohorts. Such variability cannot exist if all larvae in the cohort are equivalently affected by environmental conditions. Consequently, it was necessary to add some variation between larvae to the model.

The observations of Gallager and Mann (1986a) and Gallager et al. (1986) provide a basis for describing a range of egg sizes with a simple Gaussian function to define the frequency of a given egg size in a cohort. This range in egg sizes also produced a range in egg qualities in that larger eggs were relatively more lipid rich. The resulting simulations showed an improved fit to observation in

that a range of larval sizes and success rates at metamorphosis were obtained.

However, the range in predicted larval size and success at metamorphosis was still too constrained in comparison to observation. The obvious next option was to include a range of growth efficiencies. Genetic variation in growth efficiency is well described and may accrue from any number of processes including variations in respiration rate, protein turnover, assimilation efficiency, or feeding efficiency (e.g., Garton 1984, Koehn and Hilbish 1987, Garton & Berg 1989, Koehn & Bayne 1989, Garton & Haag 1991). In the model, respiration rate and filtration rate control growth efficiency and, although the two processes are somewhat differently affected by temperature and salinity, inserting variation in either effectively generates simulated larvae with a range of growth efficiencies. Genetic variation in growth efficiency was inserted as a range in base respiration rates using a simple Gaussian construction. This addition produced the range in outcomes at metamorphosis expected from observation.

Simulations were run to examine the influence of varying the mean of the Gaussian distribution describing egg size and respiration rate. Simulations did not change markedly with a variation in egg size because the range of viable egg sizes was tightly constrained, as discussed later. The model was more sensitive to variations in the mean base respiration rate. Here, however, simulations showed that little leeway existed for varying the central tendency of base respiration rate because substantial changes in cohort survival occurred with relatively small changes in central tendency.

These results are compared, for the most part, to observations taken under saturating conditions of food and near-optimal environmental conditions. Optimal base respiration rates, to a large extent, and egg sizes, particularly under limiting food supply, suggest that changes in the range of egg size and base respiration rate might be adaptive in certain cases that might routinely exist under field conditions. One might expect variations in respiration rate (=growth efficiency) to be the most adaptive.

Filtration Rate

Measured filtration rates always provided growth rates higher than observed. The mismatch was largest for smallest larvae. In these animals, observed reductions in neutral lipid clearly indicated that assimilation does not provide adequate resources to explain observed growth, although measured filtration rates would indicate otherwise. It seems likely that filtration rate and ingestion rate are not equivalent in larvae or that assimilation efficiency is size-dependent.

In the model, a size dependency on ingestion rate or assimilation efficiency is effectively equivalent, so no attempt was made to distinguish between the two. One might reasonably conclude that both feeding and digestion should be affected by larval development processes, particularly during early larval life, and that this might lower the amount of energy realized at a given filtration rate. One might also conclude that filtration efficiency in part is a function of the larva's use of the vellum to maintain its position in the water column as well as to feed and, so, particularly under conditions of saturating food supply where most filtration rates are measured, a tendency to filter more material than can be ingested should exist. Regardless of the cause, to lower growth rates from levels predicted from observed filtration rates, we imposed a size-dependent penalty on ingestion that was largest for the smallest larvae. The mismatch between observed growth rates and simulated growth rates from measured filtration rates, however, points to an area of early larval biology that warrants further study.

Metamorphosis

The simple biochemical construction of the model required a simple explanation for the metabolic basis for triggering metamorphosis. A full explanation of how endogenous and exogenous factors control metamorphosis (e.g., Coon & Bonar 1986, Fitt et al. 1990, Berias & Widdows 1995) does not exist. Accordingly, the approach used was derived within the limitations imposed by the four-pool biochemical construct of the model and five observations in the literature that directly related to it. (1) Filtration rate drops in larvae of about 250 μm and larger, probably due to changes in either behavior or the beginnings of tissue reorganization that must presage metamorphosis. The former option would be sufficient. Older larvae spend more time near the bottom (e.g., Dekshenieks et al., 1997) and, thus, may spend less time filtering, a fact that would be interpreted in experiment as a decline in filtration rate. The reduction in feeding rate should ultimately reduce larval scope for growth, and this should have consequences concerning the decision to metamorphose. (2) Smallest size at metamorphosis is about 275 μm. This size should be somewhat larger than the size triggering the decline in filtration rate. (3) Lipid stores decline at metamorphosis. This could be a consequence of a decline in scope for growth as well as a consequence of the energy needed to reorganize tissue. (4) Literature information suggests that larvae require a certain amount of stored energy to metamorphose successfully. Although any kind of tissue constituent might provide this energy, the reliance of larvae on neutral lipid as the primary energy store suggests that the proportion of neutral lipid is a good measure of energy available for metamorphosis. (5) The quantity of lipid present in the egg influences larval survival. Thus some information on the status of neutral lipid reserves should pertain to the decision to undergo metamorphosis.

The process of metamorphosis was modeled using these five observations to generate explicit triggers for certain steps in the process, as follows. (1) Larvae were assumed to become potentially competent to metamorphose at 275 μm, following a decrease in filtration rate at 250 μm. Simulations showed that the filtration rate decline could not be set at 230 μm or 275 μm. The minimum size for metamorphosis, in most simulations, did not fall below 285 μm, so that the 275 μm size limit was rarely invoked. That is, invoking a change in filtration rate at 250 μm normally resulted in larvae metamorphosing at sizes above 275 μm. (2) Larvae were assumed to become competent to metamorphose when a daily decline in neutral lipid of a certain level occurred. Our assumption was that larvae might be expected to continue to grow and store lipid as long as a sufficiently positive scope for growth was present and this would enhance success, but that a decline in neutral lipid would reduce success. Accordingly, metamorphosis should occur when scope for growth dropped significantly below zero. The range of observed sizes at metamorphosis suggests that some process of this sort does occur. Although the decline in filtration rate at 250 μm predestined larvae to eventually reach the trigger point defined by a significant neutral lipid decline, food quantity and quality and biochemical composition can permit growth much in excess of 250 μm before scope for growth drops to substantially negative values. Typically, in the model, metamorphosis occurred at sizes of 300–330 μm, as observed in culture. (3) Larvae were

assumed to metamorphose successfully if neutral lipid supplies were adequate. Adequacy was judged as a ratio between energy stores and structural components.

We cannot evaluate how accurately the modeled mechanism for metamorphosis approaches reality, not having available an adequate understanding of the biochemistry of the process. However, the simulations reveal some interesting trends. The choice of 250 μm as the point where filtration rate declines is based on observation, but the model also indicates that this trigger is tightly constrained to this size. Neither 230 μm nor 270 μm sizes offered verifiable results. The choice of a 25% daily decline in neutral lipid triggering competency is also tightly constrained. Values of 10% and 40% did not provide results equivalent to observations. Both larval size distributions and success rates at metamorphosis varied from observations. The choice of a ≥1:1 ratio of neutral lipid to polar lipid is also tightly constrained. Values of 0.8:1 and 1.1:1 produce unrealistic size distributions and success rates at metamorphosis.

Verification of this construction for modeling metamorphosis was directed at evaluating performance in simulating four important phenomenon: (1) variations in egg quality significantly influenced success at metamorphosis, (2) variations in food quality and quantity significantly influenced success at metamorphosis, (3) larval life span as predicted was well within the range of observations, and (4) larval size structure at metamorphosis was well within the range of observation. Obtaining these four results requires a reasonably accurate rendition of growth and survival at the biochemical resolution of the model. This suggests that the approach to modeling metamorphosis must reflect, in some significant way, the process as it actually proceeds in the larva.

Consequences of Model Construction

Larval success is determined by intrinsic and extrinsic factors. Intrinsic factors include egg size and quality and genetic makeup. Extrinsic factors include temperature, salinity, food quality, and food quantity.

Implications of Egg Size

Oyster eggs are about 50 μm in diameter, with a size range typically of 40–60 μm. The model identifies viable egg sizes in the range 37–73 μm, very similar to observations. Egg sizes outside this range are predicted to be nonviable due to lipid imbalances in early larval life. Very likely, the lower limit of 37 μm represents a packaging problem. Egg size is simply too small to provide adequate resources for the structural changes required in forming the first larval stage. In the model, the required structural tissue ratios cannot be achieved and still provide any neutral lipid reserves. In effect, the larva is never born. The upper limit of about 73 μm yields a larva that has insufficient neutral lipid reserves to cover metabolic needs immediately post-hatch. During this time, feeding is inefficient, and some of the larva's carbon needs for growth and tissue maintenance must be met by using neutral lipid reserves. The larger larvae, coming from eggs >73 μm in diameter, essentially starve to death before they can become competent filter feeders. This may provide one explanation for the small size of most planktotrophic eggs.

Presumably, the upper limit on egg size could be extended by increasing neutral lipid reserves; however, the bet hedging mode of life (e.g., Stearns 1976) would limit the amount of energy invested in any one embryo. The trade-off between additional energy expenditure and increased success at metamorphosis is clearly indicated in Figures 11, 15, and 23. Larger eggs yield successful larvae over a much larger range of respiration rates and environmental conditions than do smaller eggs. Larger eggs yield larvae that reach metamorphosis faster (shorter planktonic time), thus minimizing loss to predation and the chance of reduced survival from transient reductions in food supply. Thus, the simulations suggest that the average egg size of 50 μm minimizes the chance of reproductive failure, which increases rapidly at smaller egg sizes, while still permitting the spawning of a large number of eggs. As an example, increasing average egg size to 60 μm reduces total egg output by 31% at a given total energy expenditure. An equivalent increase in larval success is not achieved in our simulations.

The model also indicates, however, that transient reductions in food supply during larval life may increase the success rate for large eggs relative to small eggs. In this circumstance, the extra energy required to produce large eggs may be better repaid. Whether an increase in fitness is adaptively advantageous requires a better understanding of food supply under field conditions and how this influences larval survival.

Respiration (=Growth Efficiency) Effects

In the model, varying respiratory rate is equivalent to varying growth efficiency. Larvae with high growth efficiency have low respiration rates. The model identified an upper and lower limit to growth efficiency under defined environmental conditions. The upper limit varies widely depending upon environmental conditions, whereas the lower limit is relatively fixed. Simulations show that the upper limit on base respiration rate (e.g., ~1 kJ day^{-1} in Fig. 9) is determined by the point at which larvae cannot acquire sufficient neutral lipid stores to successfully metamorphose. Smaller eggs are also less viable because insufficient neutral lipid can be stored to cover larval needs over a few days post-hatch. Interestingly, very low respiration rates also normally result in unsuccessful larvae. These animals put too much assimilated carbon into somatic structural tissue and so have insufficient neutral lipid reserves. We are unaware of experimental data upon which to verify this last result.

Condition and Mortality

Many models do not explicitly follow length and weight independently (e.g., Powell et al. 1992, Dekshenieks et al. 1993). In bivalves, tracking condition permits observation of larval performance during periods of low food supply. This requires tracking length and weight independently such that not all increases in weight result in changes in length and such that no decreases in weight result in decreases in length. The performance of the model was evaluated under conditions of food deprivation by simulating the process of starvation. Although the mechanisms of death under these conditions are described in the model, death occurs due to a variety of biochemical imbalances, depending upon the initial status of the larva. Whether such a degree of complexity actually exists requires more information on the changes in larval biochemical composition under conditions of low food supply. However, the higher-level effects that integrate biochemical processes

were simulated by the model, including a decrease in weight (condition), a drop in neutral lipid content, a nonlinear time-dependent increase in mortality, and the still-successful metamorphosis of older larvae.

In addition to inadequate food supply, larvae can die if food of inadequate composition is ingested. Thus, rigorous criteria were set for biochemical compositions not allowed in viable larvae. Food having inadequate lipid or being too protein-rich resulted in mortality, even if the quantity of food remained high. These parameterizations describing mortality under such conditions are essentially *ad hoc* constructs (literature observations not being available), but they did produce cohort mortality rates that appeared to be realistic.

Effect of Diet

Most experimental studies on *C. gigas* larvae have used food supplies of ≥ 2 mg L^{-1}. This level of food saturates feeding and, in fact, raising food quantity from 2 mg L^{-1} to 4 mg L^{-1} in the model has little influence on simulated larval success. However, as in *Crassostrea virginica* (Dekshenieks et al. 1993), food quantities below 1 mg L^{-1} dramatically restrict larval growth and survival. As food supply declines, animals with high growth efficiencies are selected for in the model. At high food content, larger eggs with lower growth efficiencies also survive to metamorphosis. With rare exceptions, small eggs with low growth efficiencies never do. Thus, the influence of growth efficiency is nonrandomly distributed across egg size, and the influence seems to be mediated in part by food quantity and to a larger measure by food quality.

The influence of food content on *C. gigas* larval growth and survivorship has received considerable attention (e.g., Wilson 1978, Waldock & Nascimento 1979, Helm & Laing 1987). Although not all studies agree, low-protein diets and high-lipid diets often show improved growth and survivorship. The simulations show the positive effect of a low-protein diet on larval growth and survivorship. With this diet, a relatively larger portion of ingested energy is allocated to energy stores that in turn sustain the larva through metamorphosis. With a high-protein diet, larvae grow too fast and fail to store enough energy to sustain them through metamorphosis. The destination of protein within the larva is limited in terms of building tissue and covering metabolic needs (Table 2) if insufficient carbohydrate is ingested. Any transfer of excess amino acid into other tissue components is not permitted. Potentially, this allocation of ingested protein is too simplistic, although the simulations do provide some insight into the value of a low-protein diet.

Simulations with no neutral lipid gave similar results in terms of larval survivorship and growth. Thus, the relative amounts of protein and neutral lipid in larval food are important determinants of growth and survival.

A number of studies have identified specific components of the lipid pool as important dietary constituents (e.g., Thompson et al. 1994, 1996). The model could be expanded to track more complex biochemical pools such as polyunsaturated fatty acids (PUFAs) or sterols. The fact that the model achieves realistic simulations over a relatively wide range of environmental and dietary conditions indicates that the approach used to model larval biochemistry, including the subsuming of a diversity of lipid compounds into two pools, polar and neutral, is sufficient to provide realistic simulations of larval growth, metamorphosis and survival.

Temperature and Salinity

C. gigas is known to be relatively stenotopic for the genus. Temperature and salinity conditions describing optimal growth circumscribe a narrow range. The model reproduces this behavior. In this contribution, most simulations were run under optimal conditions of 25 C and 30‰. Lower temperatures result in insufficient neutral lipid storage and metamorphosis because feeding rate is low. Temperatures above 30 C result in biochemical imbalances due to high respiratory demand. Low salinity also results in insufficient food ingestion to meet the demands of metamorphosis. Again, data to verify the accuracy of predicted cause and effect on biochemical composition are not available.

Growth rate is a complex function dependent upon the balances of ingestion and respiration. The ability of positive environmental conditions to offset a reduction in food supply and vice versa depends upon the relative scaling of their affects on respiration and ingestion. Thus, increased temperature can "spare" a reduction in food, permitting the same growth rate, if the influence of temperature on ingestive processes scales with a larger exponent than the influence of temperature on respiration. The importance of differential scaling in the energy balance of bivalve molluscs and other animals is well known (e.g., Newell et al. 1977, Powell et al. 1992, Brown et al. 1993). Given the sensitivity of growth and survival to decreases in food supply, the fact that a decrease in food supply often occurs during summer months in *C. gigas* habitat (Kobayashi et al. 1997, Hyun et al., in press), when an increase in temperature is likely to be of significance in increasing ingestion rate, suggests that the differential scaling of ingestive processes and respiration is likely of significance for the reproductive success of the species.

CONCLUSIONS

A model that simulates the growth, development, and metamorphosis of *Crassostrea gigas* larvae has been developed. The model is the first of its kind in that it (1) tracks length separately from weight so that changes in condition can be followed and (2) predicts growth from the ingestion and transformation of biochemical constituents, thus permitting the simulation of the effects of changes in food quality. Food quality and feeding rate are important constraints in larval culture, so the model might be used to optimize culture conditions for *C. gigas* larvae as well as to investigate the influence of critical periods of food supply in larval development in the field. Of particular importance is the investigation of "teleconnections" during larval life in which events occurring at one point in larval life have consequences at another, temporally distant, point. The model has a crude depiction of the biochemistry of *C. gigas* larvae. However, the model works well even with this limited biochemistry and indicates that the formulation of sophisticated biochemically based models offers the promise of substantially improving the population modeling of marine larvae.

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BEHAVIORAL RESPONSES OF VELIGER LARVAE OF *CRASSOSTREA GIGAS* TO LEACHATE FROM WOOD TREATED WITH COPPER-CHROME-ARSENIC (CCA): A POTENTIAL BIOASSAY OF SUBLETHAL ENVIRONMENTAL EFFECTS OF CONTAMINANTS

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ABSTRACT This study examined effects upon swimming behavior of larval oysters (*Crassostrea gigas*) caused by leachates from timber treated against marine borer attack using CCA (copper, chromium and arsenic bound to the lignocellulose of the wood cells). Early veliger stage larvae were observed to avoid a layer of concentrated leachate. No effect of more dilute leachate on swimming behavior of 2-day-old larvae was detected, but 3- and 7-day-old larvae swam two to three times faster in leachate than in plain seawater and moved up and down more in leachate. The concentrations of leachate used in these experiments considerably exceed likely environmental levels, but behavior could be used to assay improvements to the treatment process and to provide data for models of the impact of treated wood structures. The methodology offers a bioassay for assessment of sublethal effects of toxicants.

KEY WORDS: CCA, veliger, swimming behavior, bioassay, copper, chromium, arsenic

INTRODUCTION

Veliger larvae of bivalves display patterns of swimming behavior that maximize their chances of remaining within the optimum region of the water column for feeding and dispersal. They swim up vertically orientated helices, intermittently ceasing swimming and sinking before resuming upward swimming (Cragg, 1980). Changes in swimming velocity and the frequency of alternation between swimming and sinking occur in response to environmental variables such as salinity, temperature and pressure (Hidu & Haskin 1978, Cragg 1980, Tremblay & Sinclair 1988, Mann et al. 1991, Dekshenieks et al. 1996). Responses to sublethal levels of toxicants have also been reported. Veligers of *Crassostrea gigas* show a greater tendency to swim near the surface of containers in water contaminated with mercury (His et al. 2000) or in water overlying contaminated sediment (Van den Hurk 1994). Contaminants may also affect overall levels of activity of bivalve veligers in experimental chambers (Chang et al. 1996, Thompson et al. 1997). In their review of bioassays using bivalve larvae, His et al. (2000) draw attention to the potential of using swimming activity in bioassays as a means of detecting sublethal effects of toxicants. The current study was designed to develop such a bioassay. It was performed as part of a larger investigation designed to evaluate the effects of leachate, from biocide-treated wood, on the marine environment (Cragg 1995).

To protect submerged wood in marine structures from rapid and severe biodegradation (mostly due to boring invertebrates) it is impregnated under pressure with biocides such as copper-chrome-arsenic (CCA) (Eaton & Hale, 1993). After treatment and prior to drying, the components of CCA are allowed to react with the lignocellulosic components of the wood to form insoluble compounds, a process known as fixation (Anderson et al. 1991. Though the treatment is resistant to leaching, detectable amounts of the elements in the treated wood enter the environment when placed in seawater (Hayes et al. 1994, Lebow et al. 1999). In recent years, evidence has been presented that in marine environments, leachate from CCA-treated wood can have a range of effects on non-target species dwelling on or near the wood (Weis & Weis 1992, Weis & Weis 1993, Weis et al. 1993). However, other field studies using bioassays suggest that in environments with sufficient tidal water exchange, effects are likely to be short-lived and localized (Wendt et al. 1996). The mathematical model of Brooks (1996) also highlights the importance of taking account of water exchange rates when predicting environmental effects of leachate from CCA-treated wood. The value of such models is dependent on adequate information about the toxic effects of particular concentrations of toxicants.

Measuring total element concentrations alone is not enough to predict biological effects, and bioassays give a better measure of the bioavailability of the element (Chapman & Long 1983). Furthermore, single metal studies are not adequate for predicting the effect of a leachate that may contain three toxic elements, but bioassays permit the detection of any synergistic effects between elements. Field observations have shown that the presence of elevated levels of the elements investigated in this study may modify settlement behavior of invertebrates (Weis & Weis 1992, Brown et al. 2000). In this study the potential of these elements to modify swimming behavior was investigated. This article summarizes findings from a number of experiments performed in order to determine optimum conditions for a bioassay using swimming behavior as the measurable response.

MATERIALS AND METHODS

Preparation and Analysis of Leachate

Sapwood blocks of Scots pine (*Pinus sylvestris* L.) with unsealed crosscut faces were impregnated with copper-chromium-arsenic (CCA) solutions by the Bethell vacuum-pressure process (Eaton & Hale 1993). The CCA solutions were made from formulations containing these elements either only as oxides or as salts (Celcure AO and Celcure A respectively, Rentokil UK). Retention of CCA was calculated from the gain in block weight due to solution uptake and the concentration of solution used. The treated wood was kept in non-drying conditions for at least seven days to permit fixation reactions between the lignocellulose complex of the wood and the treatment chemicals to take place. The blocks were then dried to below fiber saturation point. Blocks used for

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preparation of leachate were selected from those with solution uptakes typical for well-treated wood.

For initial experiments, leachates were prepared by grinding wood blocks (50 × 30 × 25 mm, untreated or treated to retentions of 12, 26 and 47 kg m^{-3} CCA) to sawdust then agitating the sawdust frequently by hand for seven days in 250 ml of seawater at room temperature. Leachates produced from 6 blocks of the same original retentions were pooled prior to use in experiments. These leachates were obtained under conditions that were intended to generate unnaturally high leachate concentrations to first establish whether a swimming response was detectable.

Leachates for the main experimental series were generated in a manner that more closely resembled processes that occur under field conditions. They were prepared by immersing treated and untreated blocks in agitated seawater of approximately 34‰ (obtained from Langstone Harbour, Portsmouth, UK) for 24 hours at 24 ± 1°C. The volume of seawater used varied according to the size of block (Table 1). Agitation was achieved by means of an orbital shaker operating at 120 rev min^{-1} or a magnetic stirrer. After 24 hours of agitation the leachates were retained for experimental use (these are referred to in the subsequent account as 1st day leachates) while the wood blocks were resubmerged for a further 24 hours in fresh filtered seawater to produce the 2nd day leachate. Full details of conditions for leachate production are summarized in Table 1.

A 50-ml sample of each leachate and of uncontaminated seawater was acidified with 2 ml of concentrated Analar grade nitric acid. The leachate samples were analyzed for concentrations of copper and chrome using atomic absorption spectrophotometry (AAS) with polarized Zeeman background correction and an acetylene/compressed air flame. The average of the two measurements that were taken per sample to the nearest 0.01-ppm are presented in Table 1. The analytical method proved incapable of providing reliable measurements of arsenic concentrations.

Larval Rearing

Initial experiments were performed using larvae of *Crassostrea gigas* (Thunberg), ranging in lengths between 90–128 μm, taken from cultures at the IFREMER Mus de Loup laboratory.

For the main experimental series, two- and three-day-old larvae of *C. gigas* were obtained from separate fertilizations with gametes stripped from adults supplied by Guernsey Sea Farms. In each case, gametes were obtained from a single pair of adults. The embryo production methodology for UK Environment Agency toxicity assessments (Anon, 1996) was used. The zygotes were reared without food, in seawater from the hatchery that supplied the adults. The unfed D-shaped veliger larvae, which were 48h-old at the time filming began, measured 80–90μm in length while the 72h-old larvae measured 100–120 μm.

A third set of *C. gigas* larvae were shipped overnight by courier from Seasalter Shellfish Hatchery, Cumbria, U.K. The larvae arrived wrapped in chilled moist filter paper and were slowly warmed to room temperature then resuspended in aerated, filtered Langstone Harbour seawater. The water temperature was then maintained at 24 ± 1°C—the hatchery rearing temperature. At the time filming of these larvae began, they were seven days old and measured 170–180 μm in shell length.

Observations of Larval Swimming Behavior

In the initial experiments, larvae in 3.5 ml of seawater taken from rearing tanks were placed in a 5-ml cuvette and left five minutes to acclimate. Swimming behavior was filmed, under normal laboratory lighting, within a marked 5-mm vertical distance for five minutes prior to the gentle addition of a 20, 50 or 100μl sample of leachate to the water surface. Swimming activity over 5 minutes was recorded using a video camera with a macro zoom lens.

In the main experimental series, acrylic 4.5-ml spectrometry

TABLE 1.

Details of age of *Crassostrea gigas* larvae observed and of the concentration of copper and chromium (ppm, mean ± SD) in seawater to which they were exposed. The seawater was either obtained from the oyster hatchery or was locally derived and in some cases modified by leachate from the first or second day of immersion of a wooden block with or without treatment by salt or oxide formulation of CCA.

Age of larvae	Seawater type	Leaching period (1st or 2nd Day)	Leaching water volume (ml)	Dimensions of leached block (mm)	Treatment of leached block**	Element concentration	
						Cu	Cr
2 Days	Hatchery Water	None	300	40 × 20 × 20		<0.01	0.18 ± 0.01
	Leachate	1st	300	40 × 20 × 20	None	<0.01	0.19 ± 0.01
	Leachate	2nd	300	40 × 20 × 20	None	<0.01	0.22 ± 0.01
	Leachate	1st	300	40 × 20 × 20	50kg.m^{-3} oxide	0.17 ± 0.01	0.22 ± 0.03
	Leachate	2nd	300	40 × 20 × 20	50kg.m^{-3} oxide	0.08 ± 0.01	0.21 ± 0.01
	Leachate	1st	300	40 × 20 × 20	55kg.m^{-3} salt	<0.01	4.19 ± 0.005
	Leachate	2nd	300	40 × 20 × 20	55kg.m^{-3} salt	<0.01	1.49 ± 0.01
3 Days	Hatchery Water	none				<0.01	0.17 ± 0.01
	Leachate	1st	200	40 × 20 × 20	None	<0.01	0.17 ± 0.01
	Leachate	2nd	200	40 × 20 × 20	None	<0.01	0.17 ± 0.01
	Leachate	1st	300	60 × 39 × 20	31kg.m^{-3} oxide	0.62 ± 0.01	0.24 ± 0.01
	Leachate	2nd	300	60 × 39 × 20	31kg.m^{-3} oxide	0.07 ± 0.01	0.19 ± 0.01
7 Days	Local Seawater					<0.01	<0.01
	Leachate	1st	800*	60 × 39 × 39	23kg.m^{-3} oxide	0.19 ± 0.01	0.11 ± 0.01
	Leachate	2nd	800*	60 × 39 × 39	23kg.m^{-3} oxide	<0.01	0.04 ± 0.01

* Stirrer used (otherwise agitation by shaker during leaching)

** CCA uptake expressed as kg dry salt or oxide per cubic metre of wood

cuvettes (ELKAY ultra VU) were filled with 2 ml of leachate and 2 ml of the seawater in which the larvae had been reared. This contained sufficient larvae to provide subjects for filming without causing abnormal behavior due to overcrowding. Larvae were exposed to first or second day leachates and compared to control larvae swimming in cuvettes containing seawater without leachate. Five replicate cuvettes were prepared for each treatment. Details on types of leachates investigated are given in Table 1.

The cuvettes were illuminated with a fiber-optic source from the side and larval behavior was filmed against a black background using a color video camera (JVC TK1381) with a macro zoom lens. The lens was zoomed and focused so that either the whole length of the cuvettes was visible, or that the width of the visual field was filled with two adjacent cuvettes. The recorded image included a scale for measurement of parameters of larval swimming paths. Larvae were acclimatized to conditions in the cuvette for 30 minutes prior to recording two-minute film for each cuvette. Further filming of the same cuvettes was conducted at intervals for up to one day. Filming was conducted at 22 ± 2°C.

Measurements of the following three variables were made from projections of the video recordings on a flat screen:

1. the total number of swimming larvae;
2. the percentage of the total number of larvae within the cuvette ascending and descending per minute through a horizontal line 20 mm from the bottom of the cuvette;
3. the vertical velocity over 1 mm of ascending larvae.

Statistical Analysis

Data relating to larvae of different ages were analyzed separately as the leachates used at different ages were not directly comparable. Transformed data on activity and velocity were analyzed by GLM using adjusted SS in the tests. Plots of residuals and Levene's test for heterogeneity of variance revealed no evidence of departure from the assumptions of ANOVA tests. Tukey or Tukey Kramer tests were applied to pair-wise comparisons to determine the sources of significant differences in the factors. Activity data were transformed by taking square roots of arcsin transformed percentages and analyzed in a model using leachate type and period of leaching as fixed factors. Velocity data were transformed by taking natural logs and analyzed in a model using type of leachate as a fixed factor and cuvette replicate as a random factor. All statistical tests were performed with Minitab version 12.1 (Minitab Corp.)

RESULTS

Copper concentrations were below levels of detection (<0.01 ppm) in hatchery water, Langstone Harbour seawater and leachates from untreated wood blocks. Chromium concentrations ranged between 0.17 and 0.24 ± 0.01 ppm in all samples that contained hatchery water. Concentrations of Cu and Cr in leachates from treated wood were generally lower in 2nd day leachates than in 1st day leachates, but there was no clear correlation between CCA retention, block surface area or leaching volume and metal concentration in the leachate (Table 1). Much higher levels of chromium were found in the leachate from wood treated with the salt- than the oxide-type CCA.

In the initial experiments, larvae were observed to swim in a normal vertical helical pattern within the cuvettes. Those encountering a surface leachate layer derived from the addition of 100 µl of leachate obtained from blocks treated to 47 kg m^{-3} CCA stopped then sank before resuming upward swimming. During the 5-minute filming period, their activity decreased until, at the end, most larvae were inactive. Samples of leachate from blocks treated to 12 and 26 kg m^{-3} CCA produced less pronounced effects, although larval swimming activity still decreased over time. No effect on normal helical swimming behavior was detected within the filming period, in cuvettes where leachate from untreated wood was added.

In the main experimental series, leachate type had a significant

TABLE 2.

Effect of different types of leachate on activity of *Crassostrea gigas* veligers of three different ages. Analysis of variance using leachate type and leaching period as fixed factors, with percentage activity data transformed and using adjusted SS for tests.

Source	DF	Seq SS	Adj SS	Adj MS	F	*p*
2-day larvae						
Leachate type	3	17.57	17.57	5.86	3.78	0.020
Leaching period	1	0.75	0.75	0.75	0.48	0.493
Type × period	3	4.61	4.61	1.54	0.99	0.410
Error	32	49.64	49.64	1.55		
Total	39	72.57				
3-day larvae						
Leachate type	2	35.14	35.14	17.57	12.09	<0.0005
Leaching period	1	0.09	0.09	0.09	0.06	0.802
Type × period	2	1.75	1.75	0.88	0.60	0.556
Error	24	34.88	34.88	1.45		
Total	29	71.85				
7-day larvae						
Leachate type	1	29.25	29.25	29.25	11.16	0.004
Leaching period	1	0.63	0.63	0.63	0.24	0.630
Type × period	1	0.78	0.78	0.78	0.30	0.592
Error	16	41.95	41.95	2.62		
Total	19	72.61				

TABLE 3.

Effect of different types of leachate on swimming velocity of *Crassostrea gigas* veligers of three different ages. General linear models used with velocity data log transformed, cuvette replicate as a random factor, leachate type as a fixed factor and adjusted SS for tests.

Source	DF	Seq SS	Adj SS	Adj MS	F	*p*
2-day larvae						
Leachate	3	0.38	2.67	0.89	1.14	0.360*
Cuvette	16	14.65	14.65	0.92	3.47	<0.0005
Error	112	29.52	29.52	0.26		
Total	131	44.56				
3-day larvae						
Leachate	2	30.35	28.50	14.25	24.79	<0.0005*
Cuvette	12	7.26	7.26	0.60	4.26	<0.0005
Error	195	27.68	27.68	0.14		
Total	209	65.29				
7-day larvae						
Leachate	1	33.69	33.54	33.54	29.21	0.001*
Cuvette	8	9.50	9.50	1.19	7.6	<0.0005
Error	217	33.91	33.91	0.16		
Total	226	77.11				

* Not an exact F-test.

effect on levels of activity in 2-, 3- and 7-day larvae, but no difference could be detected between the effect of 1st and of 2nd day leachate (Tables 2 and 3). Three- and 7-day-old larvae exposed to CCA-oxide leachate were two to three times as active ($p < 0.005$) as those in water not exposed to treated wood (Fig. 1). Most 2-day-old larvae concentrated near the surface, so activity recorded lower down cuvettes was low. Differences between activities in the different types of leachate of these larvae were small but, in the case of the difference between activity in leachate from CCA-oxide treated wood and that in leachate from untreated wood, significant ($p = 0.033$).

Larvae in the cuvettes continued to be active for at least a day after initiation of the experiments, except in the case of 7-day-old larvae exposed to CCA-oxide leachate, which became totally inactive after 24h. The significantly higher activity of 3-day-old larvae was no longer evident three hours after the larvae were introduced to the leachate.

While the 2- and 7-day larvae in seawater without leachate had similar mean upward swimming velocities (0.32 and 0.31 $mm.s^{-1}$ respectively), three-day larvae were much slower (0.22 $mm.s^{-1}$) (Fig. 2). There was no significant difference between the velocities of 2-day-old larvae in seawater alone and in seawater with various leachate types, but in the case of 3- and 7-day larvae the presence of leachate resulted in significantly higher swimming velocities ($p < 0.05$) (Table 3). Three-day-old larvae subject to CCA leachate swam 1.4 times faster than those in leachate from untreated wood and 2.7 times faster than those in hatchery water. Seven-day larvae subject to CCA leachate, swam on average twice as fast as those in uncontaminated seawater. Significant cuvette to cuvette variations in mean swimming velocity occurred, accounting for 33%, 11% and 12% of variance in the case of 2-, 3- and 7-day-old larvae respectively.

DISCUSSION

The Nature of Leachates

The analytical data from the leachates indicate that the rate of leaching of copper and chromium from treated wood generally decreased with time. This is consistent with findings of other studies of leaching into seawater from CCA-treated wood (Albuquerque et al. 1996). Studies to date suggest that the rate of leaching declines exponentially and that this is true for all three elements (Brooks 1996, Cragg et al. 2001). Factors such as the retention of preservative, the surface area of wood exposed to the water, the proportion of exposed wood surface that consists of cross-cut wood cells and the volume of water used all affect the concentration of metals in the leachate (Cragg et al. 2001). In this study, the use of small leaching volumes, of a high proportion of cross-cut surfaces and of temperatures at the upper end of the range likely to occur in the environment, will have lead to concentrations of leached elements exceeding the worst case scenario proposed by Brooks (1996).

The chromium concentrations measured in the hatchery water (0.17 ppm), greatly exceed the expected chromium concentration of uncontaminated seawater of 0.003 ppm (Brooks 1993). The elevated chromium levels may indicate the presence of stainless steel somewhere in the hatchery water system. The experimental design did not permit the isolation of this effect on the larvae from other factors.

Larval Responses to Leachate

Control larvae swam at velocities somewhat lower than those reported for other oyster veligers, 0.37 to 3.10 mm s^{-1} depending on age and conditions (Mann & Rainer 1990). This may reflect the small size of the experimental chamber, but Mann and Rainer demonstrated that only at oxygen tensions less than 20% of saturation were rates of swimming significantly lower.

Toxic effects of high concentration leachates were evident in the decreasing activity of the larvae in the initial experiment and in 7-day-old larvae in the main experimental series. The initially higher swimming activity by 3- and 7-day-old larvae in the presence of CCA leachates might be due to a stimulatory effect of Cu on cell physiology, a direct physiological response, rather than a behavioral response mediated by the nervous system. Evidence that such effects may occur is provided by the increased the rate of

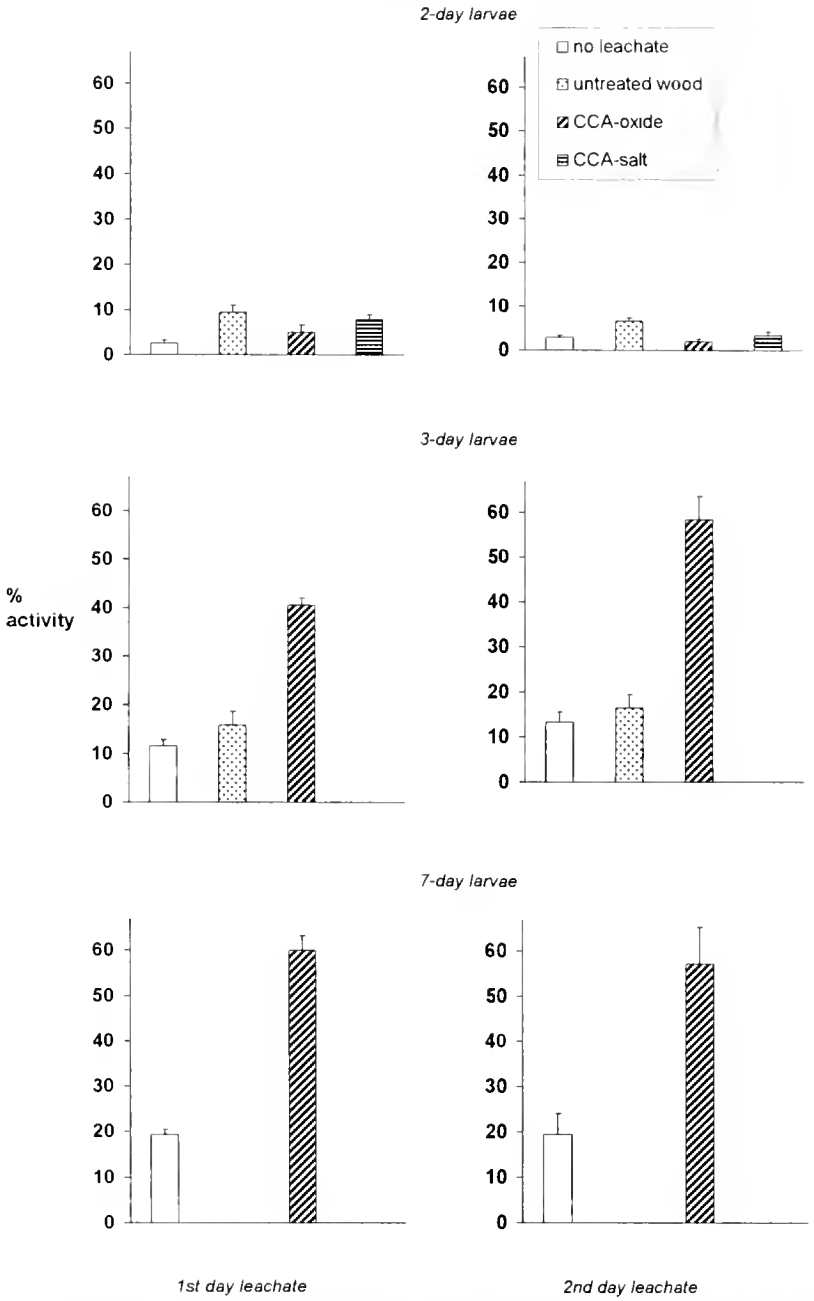

Figure 1. Activity of 2-, 3- and 7-day-old *Crassostrea gigas* veliger larvae in cuvettes containing seawater with or without leachate from wood blocks. Leachate obtained after the first or second day of immersion of the blocks. The blocks were with or without treatment by CCA salt or oxide solutions. Numbers of larvae ascending plus numbers descending past a reference line on the cuvettes are expressed as mean + SE of % of the total number of active larvae.

cell cleavage and larval growth observed by Hoare et al. (1995) in *Mytilus edulis* larvae exposed to 8 ppb of copper. However, it is more likely that the increased swimming velocity and the greater overall levels of up and down movement represent kinesis-type responses to inimical conditions. Kinesis responses may also be responsible for the increases in activity and changes in vertical distribution of larvae in response to contaminants observed by Thompson et al. (1997), His et al. (2000) and Van den Hurk (1994). Changes in velocity, activity and vertical distribution may be due to the same response. Increased swimming velocity would result in larvae reaching the surface more rapidly. A larger number of larvae at the water surface is liable to increase the rate of collision between larvae. Larvae that collide tend to perform the fright response, which involves retracting the velum, closing the shell and sinking (La Barbera 1974; Cragg 1980), then resume swimming.

By increased swimming activity, a larva will sample water quality over a greater vertical range and, due to vertical differences in current velocity, also horizontal range. The chance of being moved away from the polluting source is thus increased (Dek-

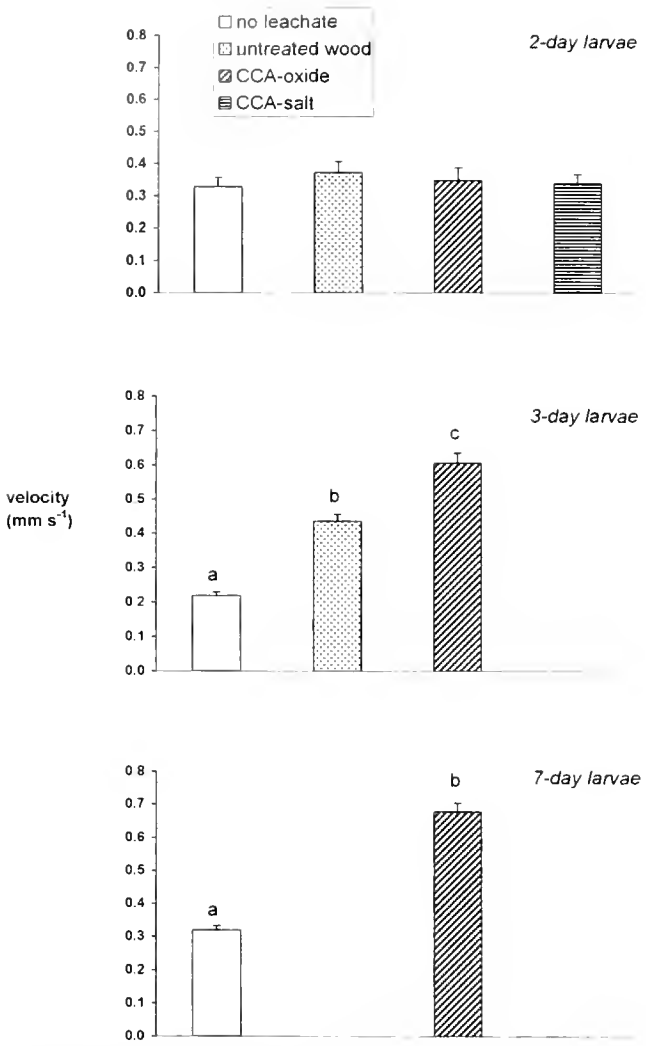

Figure 2. Vertical velocity (mean + SE) of 2-, 3- and 7-day-old *Crassostrea gigas* larvae in cuvettes containing one of four seawater types: seawater alone; seawater with leachate from untreated wood; seawater with leachate from wood treated with an oxide formulation of CCA; seawater with leachate from wood treated with a salt formulation of CCA. Leachate obtained after the first day immersion of the blocks. Significantly different means indicated by different letters (Tukey-Kramer, $p < 0.05$).

shenieks et al. 1996). Further information about the nature of the response would be obtained if larvae were kept in a deeper vessel, permitting investigation of whether larvae tended to sink further before resuming swimming.

The fact that the 3-day-old larvae swam faster in the leachate of an untreated block than in seawater alone suggests that the larvae can detect soluble chemicals derived from the wood itself. Possible candidates include soluble sugars, pectins or the stilbene, pinosylvin, a biocidal compound deposited during heartwood formation in *Pinus sylvestris* (Eaton & Hale 1993).

The lack of a detectable response in the two-day-old larvae may reflect their developmental status. The ability to close the shell appears some time after initial shell formation (La Barbera 1974; Cragg 1980) and the nerve network that innervates the ciliated cells of the velum (Cragg 1989) may not have become functional.

The apparent avoidance behavior of larvae encountering high concentration leachates, observed in the initial experiments, resembles the responses reported by Gruffydd (1976) of veligers of the scallop *Chlamys islandica* exposed to layers of low salinity seawater, and may represent a generalized response to inimical conditions. The avoidance is achieved by the fright response and is probably initiated by stimulation of a sense organ.

Potential for a Behavioral Response Bioassay

The behavioral responses observed in these experiments can be readily measured with simple laboratory equipment and thus have the potential to be used as a bioassay. Behavioral responses are of particular value as bioassays, since they occur at pollutant levels too low to produce clear toxic responses. The results of these experiments highlight the need to include controls for the effect of wood and even for source of water in the assay design. The continued activity of larvae over the day after the initiation of experiments, indicates that the cuvettes provided adequate conditions for the larvae and were thus suitable for a bioassay. By using image acquisition and image analysis software, the increased efficiency of data capture would enable a greater level of replication than in this study, increasing the precision of the assay.

This bioassay could be used to evaluate modifications to treatment procedures designed to minimize in-service leaching. Amongst the modifications worth testing would be the use of a post-treatment, pre-installation leaching in water intended for making up further treatment solutions. Our limited data and more extensive measurements reviewed by Cragg et al. (2001) indicate that rates of leaching tend to decline rapidly. By using pre-installation leaching, the highest emissions of the elements of CCA would be retained for future treatment rather than released to the environment. Modifications to the post-treatment fixation regime may also improve leach resistance, as has been shown by Lebow (1997). Furthermore, Archer et al. (1994) showed that the formulation of CCA used might affect leaching rates, a conclusion supported by the differences in leachate from the salt and oxide formulations used in this study. Minimization of leaching is particularly important where wood is used in aquaculture, such as in buchot culturing of mussels. In view of the concerns raised by Weis and Weis (1996) regarding the use of CCA-treated wood in places where water is almost stagnant, bioassay evaluation of treatment methods may assist in minimizing impacts of this essential protection for a useful and renewable construction material.

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STABLE ISOTOPE PROFILES OF *SERRIPES GROENLANDICUS* SHELLS. II. OCCURRENCE IN ALASKAN COASTAL WATER IN SOUTH ST. LAWRENCE ISLAND, NORTHERN BERING SEA

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ABSTRACT Three bivalve shells (*Serripes groenlandicus*, Greenland cockle), collected alive in the south of St. Lawrence Island, have been analyzed for the stable oxygen and carbon isotopic composition using a sequential method to trace the variation of hydrographic conditions (i.e., temperature, salinity, and oxygen isotope) of ambient seawater during the life-spans of these organisms. The $\delta^{18}O$ profiles of three shells exhibit a cyclic pattern interpreted as seasonal variation of Bering Shelf Water, which is the dominant water mass at the collection site. Discernible and distinct light $\delta^{18}O$ peaks occurred in the summer of 1991, suggesting unusual hydrographic conditions. These are attributed to the migration of Alaskan Coastal Water into the south of St. Lawrence Island during the summer. The changed oxygen isotopic compositions of seawater between 1990 and 1993 in the study area supports the idea that the flow of Alaskan Coastal Water, intensified by the enhanced freshwater discharges from the Alaskan coast, can be migrated westward into the south of St. Lawrence Island. The $\delta^{13}C$ profiles also show apparent cyclicity, reflecting the variation of available carbon isotopes in relation to photosynthesis and oxidation of organic matter. A seasonal bloom of phytoplankton and consequent oxidation of organic matter is the possible cause of the noticeably light $\delta^{13}C$ peak in 1992, not the influx of terrestrial carbon or metabolically derived carbon. The warm Alaskan Coastal Water, characterized by low oxygen isotopic composition, is clearly recorded in the shell isotope profiles, which provides a record of the hydrographic variation of the ambient seawater.

KEY WORDS: *Serripes groenlandicus*, bivalve, stable isotope, water mass

INTRODUCTION

Little attention has been paid to the stable isotopic composition of shallow marine biogenic carbonates, mainly because the extreme spatial and temporal changes of hydrographic conditions make their interpretation of compositions difficult. In contrast, exceptions are the stable isotope studies on the growth rate and longevity of mollusk shells to obtain the information on population dynamics (e.g., Tanabe 1988, Jones and Quitmyer 1996). Shells of marine bivalves, gastropods, corals, and brachiopods in the nearshore and continental-shelf environment have been the object of a number of stable isotope studies because their skeleton chemistry has the potential to record the ambient seawater conditions (summarized in Rhoads and Lutz 1980). In particular, many investigations have explored the relationship between the stable oxygen and carbon isotopic compositions of bivalve shell carbonates and environmental information using microsampling to highlight high-resolution temporal changes of ambient seawater during the lifespans of living or fossil organisms (Krantz et al. 1987, Krantz 1990, Cornu et al. 1993, Weidman et al. 1994, Jones and Allmon 1995, Klein et al. 1996, Bemis and Geary 1996, Bice et al. 1996, Kirby et al. 1998, Khim et al. 2000, Khim 2001).

The hydrographic conditions in the shallow marine environment of temperate and sub-arctic regimes vary seasonally. In winter, the water column is vertically homogeneous due to mixing resulting from the convective cooling and severe wind. On the other hand, in summer, incoming freshwater discharge and heating of the surface water result in the vertical stratification of water masses in these shallow waters. For example, in the northern Bering Sea (Fig. 1), stratification develops particularly during the summer after the sea ice melts (Coachman et al. 1975, Muench et al. 1981, Coachman and Shigaev 1992, Schumacher and Kendall 1995). The migration of water mass is of great importance because the transport and dispersal of pollutants is controlled by the coastal current in the shallow marine environment. In addition, the resultant mixing of water masses plays a potential role in influencing primary productivity by reintroducing the limited nutrients (Walsh et al. 1989).

In this paper, three living bivalve (*Serripes groenlandicus*) shells were collected in the northern Bering Sea by dredging in June 1993 during the Helix 171 research cruise. From the patterns of stable oxygen- and carbon-isotope profiles, constructed using the high-resolution sequential sampling, interesting hydrographic events can be identified distinctly.

HYDROGRAPHY AND SAMPLING SITE

The northern Bering Sea is characterized by one of the largest continental-shelf seas in the world ocean (Fig. 1a). This sea, which is the connection between the North Pacific and the Arctic Ocean, is deepening slightly toward the Bering Strait. In the northern Bering Sea lie two straits, the Anadyr and Shpanberg straits, on either side of St. Lawrence Island (Fig. 1b). Northward flows through the Bering Strait (Fig. 1a; Coachman et al. 1975, Walsh et al. 1989) result from sea-level sloping toward the Arctic Ocean (Stigebrandt 1984).

Three water masses (Anadyr Water, Bering Shelf Water, and Alaskan Coastal Water) defined by their temperature-salinity profiles have been observed in the northern Bering shelf (Fig. 1b; Coachman et al. 1975). The water masses are also characterized by the unique $\delta^{18}O_w$ (i.e., $\delta^{18}O_{seawater}$ vs. V-SMOW) values (Grebmeier et al. 1990, Cooper et al. 1997). Their results showed that the separation of Alaskan Coastal Water from Bering Shelf Water was defined by $\delta^{18}O_w$ value of about −2.0 ‰. These water masses are oriented in an east-west direction across the shelf. The most saline and coldest Anadyr Water occupies the western part of Anadyr Strait, whereas the least saline and warmest Alaskan Coastal Water occurs in the eastern part of the Shpanberg Strait. Between these lies the Bering Shelf Water of intermediate temperature and salinity. The relatively strong hydrographic boundary between Anadyr Water and Bering Shelf Water is maintained during the year, but the corresponding front between Bering Shelf Water and Alaskan

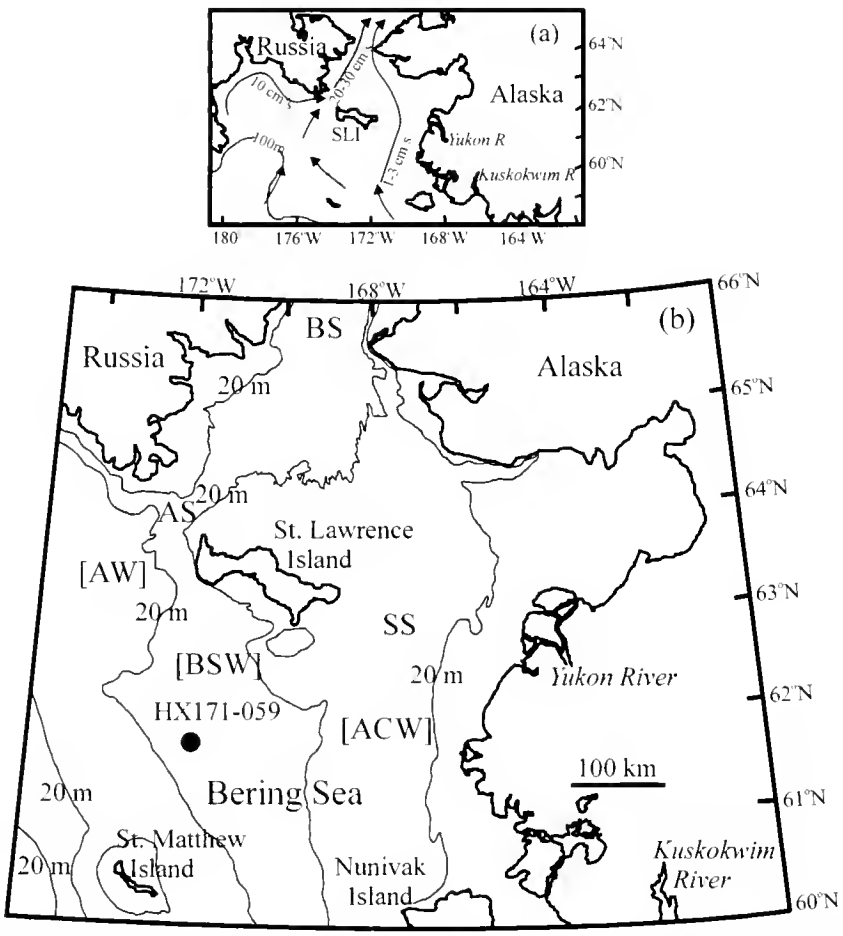

Figure 1. (a) The schematic flow pattern in the northern Bering Sea (after Schumacher and Kendall 1995). SLI: St Lawrence Island. (b) The bathymetry of northern Bering Shelf and location of sampling site (HX171-059). The three principal water masses (AW: Anadyr Water, BSW: Bering Shelf Water, ACW: Alaskan Coastal Water) are aligned in the east-west direction across the shelf. BS: Bering Strait, AS: Anadyr Strait, SS: Shpanberg Strait.

Coastal Water is usually indistinct and gradational and the lateral mixing is quite possible (Coachman and Shigaev 1992). Enhanced freshwater runoff to intensify the Alaskan Coastal Water, as well as mixing process from wind and tidal shear, works the water mass to extend its way across a front into the shelf area.

The warmer Alaskan Coastal Water (summertime bottom-water temperature: about 6°C) is characterized by a mixture of freshwater runoff with relatively saline coastal waters (Coachman 1986, Schumacher and Kendall 1995). The relatively low salinities (generally <31.8 psu, practical salinity unit) near the Shpanberg Strait are reinforced annually by the addition of Yukon River water. This marked seasonal difference in freshwater runoff causes the intensification of Alaskan Coastal Water during the warm season. Alaskan Coastal Water also shows the inter-annual variations of salinities which are the same magnitude as those of the seasonal cycle (about 0.5 psu; Coachman and Shigaev 1992).

The vertical profiles of temperature and salinity measured at the station HX171-059 (61°46.0′N, 171°16.0′W, Fig. 1b) where the bivalve shells were collected are shown in Figure 2. The water column formed a distinct two-layered stratification at the time of shell collection. High-temperature (~6.0°C) and less saline (~30.7 psu) waters form the upper layer whereas low-temperature (~0.4°C) and more saline (~31.6 psu) waters form the lower layer. A sharp thermocline lies a little deeper than the halocline, both of which maintain the water-column stability. The high temperature and low salinity of the upper layer is probably due to effects of solar radiation, and freshwater runoff and sea-ice melts, although the collection time (late June) was close to the beginning of summer when it is fairly drier. Approximately −2.0 ‰ (V-SMOW) of bottom water at the depth of 45 m is measured, which seems to be quite low, on the basis of the $\delta^{18}O_w$ definition of Bering Shelf Water (Grebmeier et al. 1990).

MATERIALS AND METHODS

Three bivalves, *Serripes groenlandicus* (Greenland cockle), were collected alive near the hydrocasting station HX171-059 (45 m deep) south of St. Lawrence Island, northern Bering Sea by dredge in late June, 1993 (Fig. 1b). This infaunal and suspended feeder is cosmopolitan in arctic and boreal regions from the subtidal zone to about 100 m. The shell of this species is composed entirely of aragonite and grows up to 10 cm as a ten-year-old adult (Andrews 1972). These features and previous research (Khim et al. 2001, submitted) suggest that *S. groenlandicus* would be useful for assessing seasonal and inter-annual hydrographic variability of the Alaskan Coastal Water from shell isotope profiles.

The growth patterns of bivalve shells with their repeated formations of annual increments provide information determining age

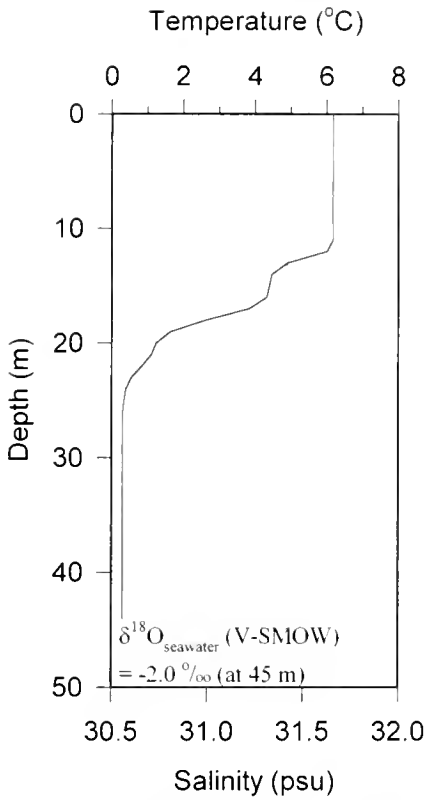

Figure 2. Vertical profiles of temperature and salinity measured at the station of HX171-059. The two-layered stratified water-column is clearly maintained by the strong thermocline and halocline that lie between 10 and 20 m. The $\delta^{18}O_w$ of bottom water (water depth of 45 m) is about −2.0 ‰ (V-SMOW) (Cooper unpubl.).

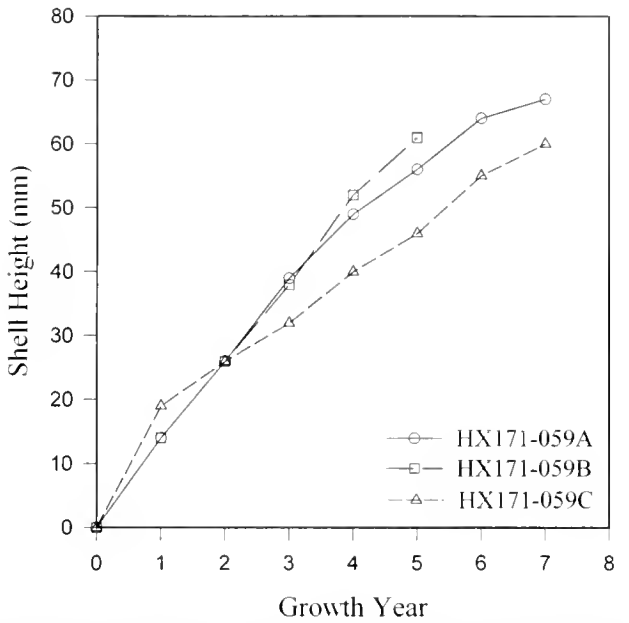

Figure 3. Growth rate of *S. groenlandicus* estimated by measuring shell height, based on the annual increments. The three shells show their different growth rate in spite of living in adjacent habitats.

and growth rates of marine bivalves (Rhoads and Lutz 1980, Jones and Quitmyer 1996). The bivalve *S. groenlandicus* produces annual growth marks which are correlated to its isotopic record (Khim et al. 2001). Figure 3 shows the growth rate history of analyzed shells using growth marks. Since the shell specimens were collected alive in 1993, calendar years may be related to the sampling intervals by counting growth bands backward from the shell margin. Consecutive sampling for isotopic determinations of shells is schematically shown in Figure 4. No sampling was done near the umbo (younger ages) because of the thin outer shell layer and near the shell margin (older years) because of the very slow growth rate.

Preparation technique for stable isotopic analyses was taken from Krantz et al. (1987). The specimens were soaked in a 5% sodium hypochlorite solution and rinsed in distilled deionized water to remove the organic matter from the shell exterior. One valve of the mollusk specimen was radically cut along the axis of maximum growth. A serial shell-sampling technique produced a set of individual carbonate powders (approximately 2 mg), which were collected in ontogenetic sequence from the outer shell layer with a 0.3-mm dental drill bur. Care was taken to avoid the penetration of the inner nacreous layer since these boreal bivalves have a thin outer layer. Isotope measurements were carried out at the University of Maine. The number of samples obtained from the specimens (HX171A, HX171B, and HX171C) are twenty, twenty-three, and twenty, respectively (Fig. 4).

Stable oxygen and carbon isotopic ratios were determined using an on-line, automatic carbonate preparation device attached to a VG Isogas PRISM isotope ratio mass spectrometer. The evolved CO_2 gas was measured after reaction in 100% phosphoric acid at 90°C. All isotope values are reported in the standard δ notation of per mil (‰) unit as the ratio of $^{18}O/^{16}O$ or $^{13}C/^{12}C$ in the sample relative to a V-PDB reference (Craig 1957, Coplen 1994). Analytical precision based on NBS-19 carbonate powders yield better than ±0.10‰ for $\delta^{18}O$ and ±0.07‰ for $\delta^{13}C$.

RESULTS AND DISCUSSION

Stable oxygen- and carbon-isotope profiles of three shell specimens are illustrated in Figure 5. The vertical axis is inverted to show the low $\delta^{18}O$ values toward the top, and the horizontal axis shows the ontogenetic growth of shell from left to right. In general, based on the thermodynamic behaviors of the oxygen isotopes in the skeletal formation (Urey 1947, Epstein et al. 1953, Grossman and Ku 1986), the low $\delta^{18}O$ values of the oxygen-isotope profile reflect the high seawater temperature during summer whereas the high values represent the low temperature during winter. Isotope values for sampled years of each shell are summarized in Table 1.

All the oxygen-isotope profiles are clearly cyclic and seasonal, but their patterns are different among the shells in the same year (Fig. 5). It may be due to the different growth rate, as judged from the width of annual bands (Fig. 3). The specimen HX171A shows a fairly low amplitude of seasonal $\delta^{18}O$ variation (Fig. 5a), ranging from 0.45 to 1.22‰ (Table 1). The calculated seasonal temperature variations through simple temperature fractionation of oxygen isotopes (−0.23/°C, O'Neil et al. 1969) are about 2 to 5°C, which corresponds to the temperature of the Bering Shelf Water (Coachman et al. 1975). The shell HX171B has a small $\delta^{18}O$ variation (about 0.8 ‰) in 1990 similar to those of HX171A (Fig. 5b), but has a prominent signal of the lowest $\delta^{18}O$ value in the summer of 1991, the amplitude of which is up to about 2.2 ‰ (Table 1). For the same years, the pattern and amplitude of the profile of specimen HX171C are similar to those of HX171B with an amplitude

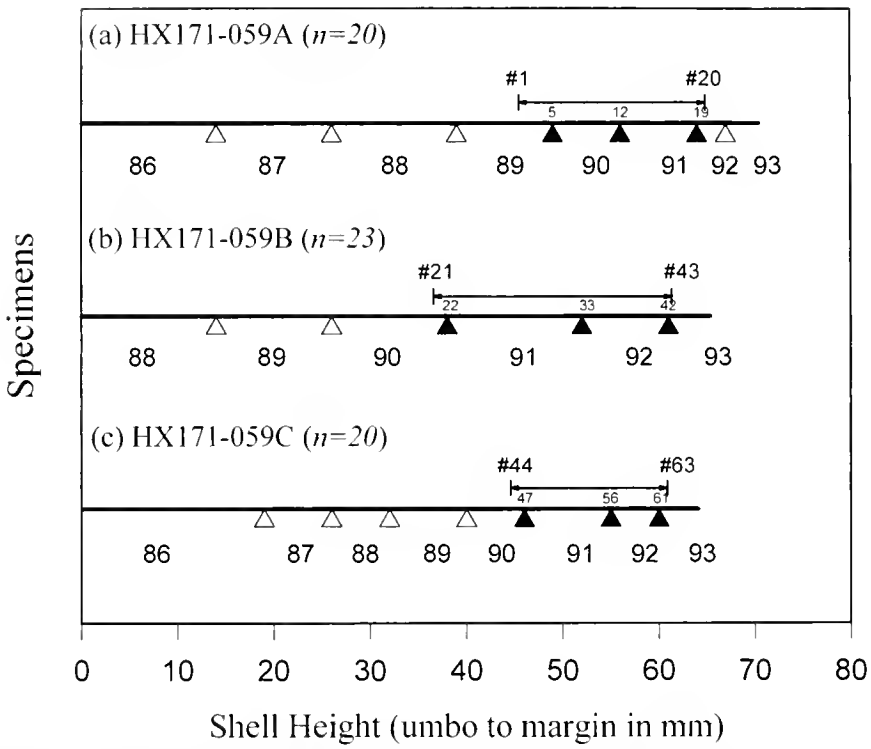

Figure 4. Sampling scheme for obtaining carbonate powders by drilling along the shell growth. The identification of annual bands along with the backward counting from margin provides the matching year for the individual isotope profile.

of 1.6‰ (Fig. 5c). Thus, the most remarkable feature of the oxygen-isotope profiles is the event in the summer of 1991 that marks the lightest $\delta^{18}O$ value obtained. A similar pattern in the oxygen-isotope profiles interpreted due to changes of hydrographic properties has been reported in mid-Atlantic coastal shells (Krantz et al. 1987). The lack of a distinct peak in the summer of 1991 for specimen HX171A is problematic. However, the most plausible explanation is that this shell lived far from the area where the two specimens (HX171B and HX171C) were obtained because the trawling area for collection was too large.

In terms of the seasonality of the $\delta^{18}O$ profiles, the bivalves can record the seasonal temperature variations during their life-spans through the isotopic fractionation of shell carbonate, under the assumption that the oxygen isotope values of ambient seawater are uniform. A variety of previous investigations substantiated that the $\delta^{18}O$ variations are controlled primarily by seasonal changes of seawater temperatures (Cornu et al. 1993, Bemis and Geary 1996, Khim et al. 2000), although the bivalve shell imperfectly records the lowest temperature due to either the growth cessation or slow growth rate during the winter (Krantz et al. 1987, Weidman et al. 1994). The $\delta^{18}O$ variation of HX171B in 1991 (about 2.2 ‰) corresponds to a seasonal temperature change of seawater of approximately 9°C, based on temperature fractionation (O'Neil et al., 1969). This seasonal temperature range is larger than expected when compared to the present-day hydrographic conditions occupied by the Bering Shelf Water (Coachman et al. 1975, Coachman and Shigaev 1992) as well as to the seasonal variations expected from HX171A (Fig. 3). Thus, at a first approximation, such seasonal temperature change in 1991 calculated from the amplitude of oxygen-isotope profile for HX171B is unlikely to occur. The large part of variation may be due to another factor to affect oxygen isotopic composition of shell carbonate when organisms lived.

An alternative plausible and potential variable to influence the oxygen isotopic composition of shell carbonates is the oxygen isotopic composition of seawater ($\delta^{18}O_w$) itself (Epstein et al. 1953, Grossman and Ku 1986). In general, the $\delta^{18}O_w$ values are linearly correlated with the salinity (Craig and Gordon 1965); the high-salinity water mass is characterized by the high $\delta^{18}O_w$ value and *vice versa*. In the northern Bering Shelf region, the Alaskan Coastal Water has lower $\delta^{18}O_w$ values than the Bering Shelf Water (Grebmeier et al. 1990, Cooper et al. 1997, Cooper unpubl.). Figure 6 illustrates the temporal variation of bottom water $\delta^{18}O_w$ values in the southern part of St. Lawrence Island. In June, 1990, the $\delta^{18}O_w$ variations demonstrate that the water masses are aligned in east-west direction and $\delta^{18}O_w$ values increase gradually westward (Fig. 6a, Grebmeier et al. 1990). Where the shells were collected in June, 1993, the increasing $\delta^{18}O_w$ trend can be similarly observed in the south of St. Lawrence Island (Fig. 6b, Cooper unpubl.). However, the absolute $\delta^{18}O_w$ values in 1993 became much lower by as much as approximately 0.5 ‰ than those in 1990. Such low $\delta^{18}O_w$ values in 1993 are attributed to the migration of Alaskan Coastal Water into this area, resulting in the drop of $\delta^{18}O_w$ value. Therefore, although $\delta^{18}O_w$ data in 1991 are unavailable in the present study, the distinct pattern of oxygen-isotope profiles demonstrates that the lightest $\delta^{18}O$ peak occurred in 1991 is presumably caused to much extent by the low $\delta^{18}O_w$ value of Alaskan Coastal Water. Krantz et al. (1987) also showed that short-term low-salinity events in the mid-Atlantic coastal area can be identified from the pattern of oxygen-isotope profiles.

The carbon-isotope profiles of live shells also show periodic patterns similar to oxygen-isotope profiles (Fig. 5). In general, the high $\delta^{13}C$ values are observed during mostly the entire season, whereas the low $\delta^{13}C$ values occur along with the low $\delta^{18}O$ values that reflect the warm season. The $\delta^{13}C$ profile of specimen

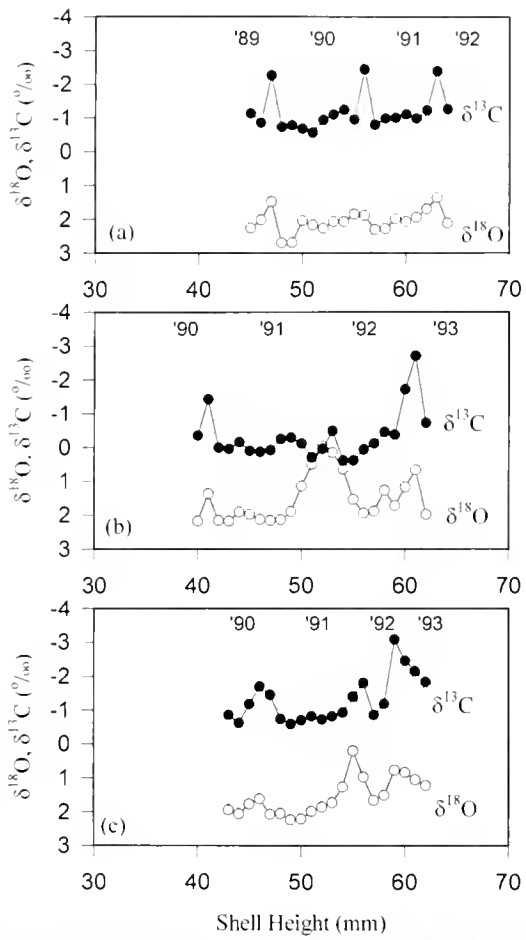

Figure 5. Stable oxygen- and carbon-isotope profiles of living bivalve *S. groenlandicus*. (a) HX171A, (b) HX171B, (c) HX171C. Note the low $\delta^{18}O$peaks in 1991 of HX171B and HX171C and the low $\delta^{13}C$ values that occurred in 1992 in HX171B and HX171C.

HX171A is cyclical with almost consistent amplitudes over the sampled interval (Fig. 5a). The mean $\delta^{13}C$ and range of variation are about −1.18 ‰ and 1.70 ‰, respectively (Table 1). The mean $\delta^{13}C$ values of HX171B and HX171C are −0.33 and −1.24 ‰, respectively, the latter of which is close to that of HX171A. The enriched $\delta^{13}C$ values of HX171B may be mainly due to the different $\delta^{13}C$ of seawater dissolved inorganic carbon (DIC) as a background level, although these shells were dredged in a same locality. The $\delta^{13}C$ patterns of HX171B and HX171C are similar in spite of different growth rates, but clearly distinguishable from that of HX171A (Fig. 5). In 1992, the light $\delta^{13}C$ peak is observed distinctly in both HX171B and HX171C, as similar event of oxygen-isotope profile in 1991. The approximate amplitude is up to 2.8 ‰ (Table 1). In addition, the shapes of oxygen- and carbon-isotope profiles of these two shells are alike in both 1992 and 1993.

Several probable variables affecting the carbon isotopic composition of shell carbonates are as follows: $\delta^{13}C$ of the DIC reservoir associated with phytoplankton productivity and reoxidation of organic matter, effects of temperature on ^{13}C fractionation, and seasonal physiological effects related to metabolic activity (Rubinson and Clayton 1969, Emrich et al. 1970, Tanaka et al. 1986, Rosenberg and Hughes 1991, Wefer and Berger 1991, Romanek et al. 1992, Klein et al. 1996). A primary factor is the available ^{13}C atoms in ambient seawater DIC (Mook and Vogel 1968), but other variables can be important. In particular, seasonal variations of the $\delta^{13}C$ values of the shell isotope profiles documented in the previous studies have been explained as a result of modification in the level of $\delta^{13}C$ of seawater DIC (Krantz et al. 1987). Due to the phytoplankton role in fractionating carbon isotopes during photosynthesis producing organic matter, the heavy carbon atoms are easily incorporated into the shell carbonate. On the other hand, isotopic fractionation through the decomposition of organic matter causes the light carbon isotopes to be assimilated in the shell carbonate (Kroopnick 1980).

The low $\delta^{13}C$ values of HX171A in summer may reflect seasonal phytoplankton productivity and decomposition of organic matter at the bottom. In the northern Bering Sea, the seasonal bloom of phytoplankton occurs during the warm months when the sea ice is retreating and the influx of nutrient-rich water is increasing (Hansell et al. 1993, Springer and McRoy 1993). In the Alaskan Coastal Water, the blooming duration may not be apparently long enough to produce a distinguishing signal in the ^{12}C atoms (Walsh et al. 1989, Coachman and Shigaev 1992). Alternatively, there is a possibility that the light $\delta^{13}C$ peaks may reflect the inclusion of metabolic light carbon formed by growing much faster during warm, favorable conditions (Rosenberg and Hughes 1991, Wefer and Berger 1991). In spite of lacking evidence to demon-

TABLE 1.

Summary of minimum, maximum and variation of stable oxygen and carbon isotope judged by the individual year over the sampled intervals.

		$\delta^{18}O$			$\delta^{13}C$		
Specimen	**Year**	$\delta^{18}O_{min}$	$\delta^{18}O_{max}$	$\Delta\delta^{18}O$	$\delta^{13}C_{min}$	$\delta^{13}C_{max}$	$\Delta\delta^{13}C$
HX171A	1989	1.47	2.69	1.22	−2.27	−0.58	1.69
	1990	1.84	2.29	0.45	−2.46	−0.81	1.65
	1991	1.35	*2.11*	*0.76*	−2.40	*−1.28*	*1.12*
HX171B	1990	1.37	2.18	0.81	−1.43	0.13	1.56
	1991	−0.04	2.16	2.20	−0.49	0.38	0.88
	1992	0.66	*1.98*	*1.32*	−2.71	*0.06*	2.77
HX171C	1990	1.62	2.24	0.62	−1.70	−0.59	1.11
	1991	0.01	1.66	1.65	*−1.80*	−0.82	0.98
	1992	0.77	*1.22*	*0.45*	−3.09	*−0.86*	*2.23*

Note: italic numerals are incomplete variation.

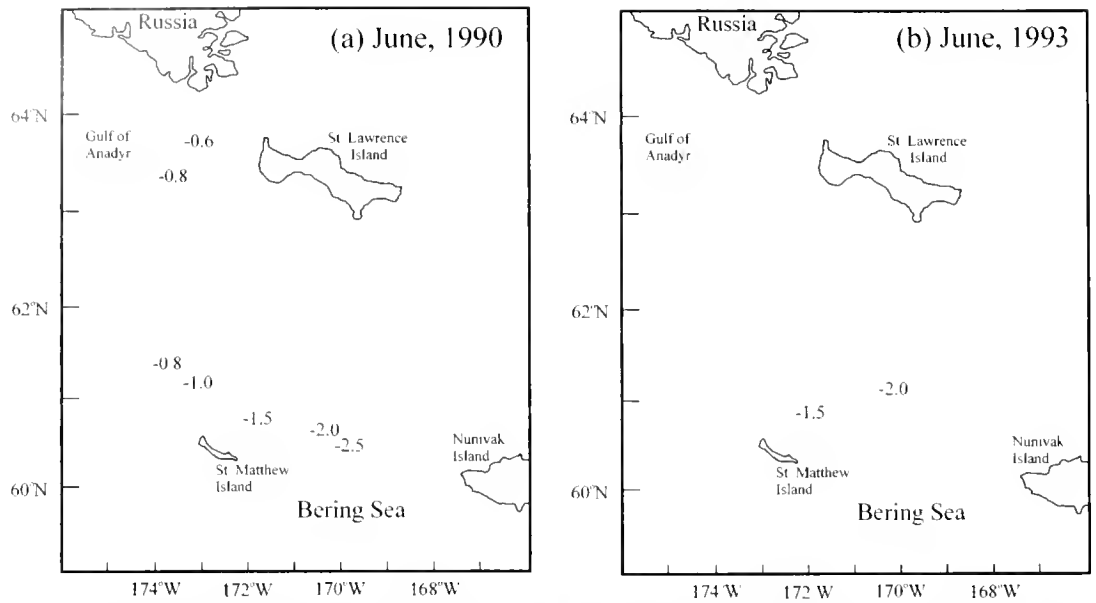

Figure 6. The spatial distribution of $\delta^{18}O_w$ values in the south of St. Lawrence Island. (a) June 1999 (from Grebmeier et al. 1990), (b) June 1993 (from Cooper unpubl.). The absolute $\delta^{18}O_w$ values in 1993 are greater by as much as approximately 0.5 ‰ compared to those in 1990. Such low $\delta^{18}O_w$ values in 1993 may be due to the migration of Alaskan Coastal Water into this area, resulting in the drop of $\delta^{18}O_w$ values.

strate that the terrestrial carbon species transported by Alaskan Coastal Water (Naidu et al. 1993), the light terrestrial carbon should be considered to interpret. Terrestrial carbon from river discharge is prominently supplied during the warm season by the intensified Alaskan Coastal Water. If the low $\delta^{18}O$ peaks observed during the summer of 1991 result from the influx of Alaskan Coastal Water, then the water mass maintains plenty of ^{16}O atoms along with low salinity caused by increasing river runoff. However, the $\delta^{13}C$ profile in that year does not show the corresponding low peaks. Thus, the addition of light terrestrial carbon during the warm season is unlikely to cause the low peak.

The distinct low $\delta^{13}C$ peak of 1992 is informative because the $\delta^{18}O$ profile has no equivalent peak in the same year (Fig. 5). As explained above, such light peak is not attributable to light carbon of terrestrial origin transported by riverine discharge. The $\delta^{13}C$ minimum in 1992 can be identified in both HX171B and HX171C, but not in HX171A. It is difficult to judge which process causes this discernible low $\delta^{13}C$ peak. The plausible mechanism resulting in this $\delta^{13}C$ peak is the abrupt change of $\delta^{13}C$ of seawater DIC by the oxidation of organic matter generated from phytoplankton blooms. A stratified water-column, a short duration of primary production on the surface layer, and a fairly shallow depth are all conditions likely to change the $\delta^{13}C$ of bottom water. Thus, the light carbon is assimilated easily in the formation of shell carbonate.

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ASPECTS OF THE GONADAL CYCLE IN THE ANTARCTIC BIVALVE *LATERNULA ELLIPTICA*

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ABSTRACT Vitellogenesis and oocyte growth in *Laternula elliptica*, a common hermaphrodite bivalve living in the soft muddy bottoms of the Antarctic continent, are reported. Formation of the gelatinous layer surrounding the oocyte, gonadic development, accumulation of mature oocytes, and spawning events were studied through histological evidence. Gonads were observed to reach maturity at a size of 49 mm. Vitellogenesis has been found to last seven months, and storage of oocytes before spawning was observed. It has been observed that once sexually mature, the animals remain with sperm cells and oocytes available during the entire year, suggesting that individuals would be prepared to spawn at any moment, probably depending on environmental conditions.

KEY WORDS: *Laternula elliptica*, Antarctic clams, vitellogenesis, oocyte growth, first maturation

INTRODUCTION

Laternula elliptica (King & Broderip 1832) is possibly the most common bivalve in the Antarctic continent wherever soft muddy bottoms are present (Powell 1965). It burrows deeply into the substrate (Stout & Shabica 1970); densities of 50 individuals per m^2 with a fresh weight biomass of 2–3 kg/m^2 have been reported (Hardy 1972).

Recently several authors have studied different aspects of the reproduction of *L. elliptica* (Pearse et al. 1986; Pearse et al. 1987; Urban & Mercuri 1998; Ansell & Harvey 1997). Several questions remain unanswered, however. This article deals with the vitellogenesis period and oocyte growth within the ovarian portion of the hermaphrodite gonad, the size and age of gonadic maturation, the formation of the gelatinous layer surrounding the oocyte, the accumulation of mature oocytes, and spawning events, studied from histological evidence.

MATERIAL AND METHODS

The material was obtained by one of the authors (G.M.) during a joint German-Argentine project (Klöser & Arntz 1995; Urban & Mercuri 1998), at the location of Potter Cove, King George Island, South Shetland Islands. A monthly sampling was conducted at a fixed station at 10 m depth (see Urban & Mercuri 1998, for locality details). Histologic slides were prepared for one complete year of sampling (1993–1994). Gonads were fixed in Bouin's solution, preserved in 70% alcohol and treated with common inclusion procedures. Sections of 5 microns were stained with Harris' hematoxylin and eosin (Merck). The ovaries of 30 individuals of standard adult bivalves (of about 65–80 mm in shell length) were studied for each monthly sample. A total number of 1113 oocytes (73–117 per monthly sample) with distinct nucleolus were measured, comprising the whole visual field.

In a later sample, in February 2000, gonads of 22 individuals of about 27–73 mm were studied with the same procedures in order to determine the size of gonadal development and maturation.

RESULTS AND DISCUSSION

Under the stereoscopic microscope it is possible to observe, in the ovarian portion of mature clams, the mature oocytes and empty spaces related to partial spawning (Figs. 1–3).

A gelatinous layer (Figs. 4–9) envelops the mature oocyte. This outer layer becomes visible (3.1 SD 1.3 microns thick) during vitellogenesis when the oocytes are 90–100 microns in diameter. Maximum thickness (13.6 SD 5.0 microns) of the gelatinous layer is reached in oocytes of 220 microns in complete diameter (these oocytes have a mean cytoplasmic diameter of 193 microns).

According to the histograms of oocyte diameter frequencies, this process of wall formation takes 3–4 months, starting in March–April and ending in September (Figs. 10 and 11). Ansell and Harvey (1997) stated that after fertilization this gelatinous envelope condensed to form a strong, sticky, elastic capsule in which further development took place. We consider this gelatinous layer to be a vitelline membrane, formed by the oocyte itself (Huebner & Anderson 1976).

The largest oocyte mean diameter we measured, including the gelatinous layer, was 220.7 microns in February 1994 (Fig. 10), but we consider the oocyte to be mature at 171.5 microns external diameter, and the mean mature oocyte is 195.2 microns (including gelatinous layer).

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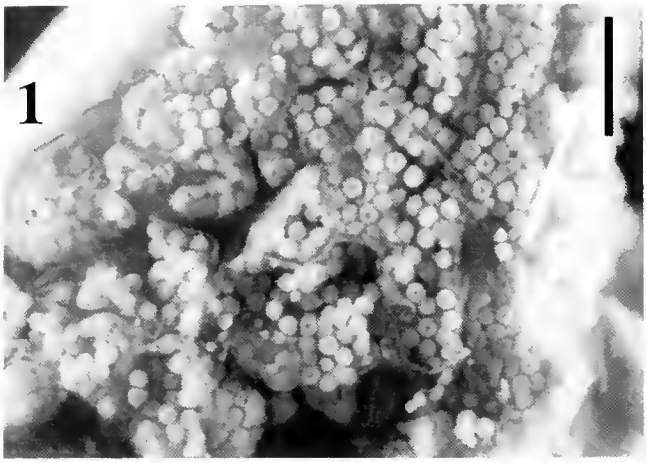

Figures 1–3. Ovarian portion of gonads of *Laternula elliptica* (>60 mm shell length) viewed through stereoscopic microscope. (1) Ripe ovary; (2) Partially spent ovary; (3) Spawned ovary with remnant oocytes. Scale Bar = 1200 microns.

The modal diameter peaks for bigger oocytes show no variation from January to July (171.5 microns). But modal diameters of the small oocytes show a remarkable constant tendency to grow from January (55.5 microns) to June (142.5 microns); this is the main period of vitellogenesis. The period of favorable light conditions to phytoplankton growth in Potter Cove appeared to be very limited, approximately 1.5 months during summer (Schloss et al. 1997). Apart from that, resuspension of benthic material and possible input of terrigenous material constitute the main carbon source in summer time, while resuspended material or secondary bacterial production would account for it during the rest of the year (Schloss et al. 1997).

We consider that, as resorption of unspawned oocytes was never observed in any case, there is currently a storage of large mature oocytes in the female follicles, ready to spawn during the entire year. Protected lecitotrophic embryos, nourished by a considerable amount of yolk reserves which enable them to avoid a free swimming larvae stage in the first phases of the development, is the reproductive mode for *L. elliptica* (Pearse et al. 1986; Bosch & Pearse 1988).

Urban and Mercuri (1998) found that ripe ovaries seem to dominate, with values between 60–80% throughout the whole year, and only during the warmest two months (February and March) spent ovaries dominated, with ripe ovaries being reduced to about 25%. The authors suggested that it is most likely that the oocyte development cycle last longer than one year. Our results conclude that the oocytes complete their growth in less than seven months, and are stored until spawn. Once sexually mature, the animals remain with sperm cells and oocytes available during the whole year, suggesting that individuals would be prepared to spawn at any moment, probably depending on environmental conditions.

In a later sample performed during February 2000 in the same study area, microscopic differentiation of both testicles and ovarian follicles was observed at a size of 27 mm. Taking into account growth rates estimated by Brey and Mackense (1997) and by Urban and Mercuri (1998), this size corresponds to individuals <2 years old. Maturity in the male portion of the gonad is evidenced by the existence of mature sperm cells at a size of 30 mm shell length (Fig. 6).

At a shell length of 32 mm, which corresponds to individuals ~3 years old, it is possible to observe some oocytes covered by the characteristic gelatinous layer (up to 140 microns in external diameter). Ovaric maturity might then be reached when the individuals attain a size of 49 mm shell length, which corresponds to an age of approximately 4 years. It is at this stage that the gelatinous layer containing oocytes of 170 microns external diameter can be observed.

We conclude then that *L. elliptica* is a simultaneous hermaphrodite, completely mature at the age of 4 years. Information is still needed about its first stages of development, in order to assess the possibility of early development of any of the gonadic portions relative to the others.

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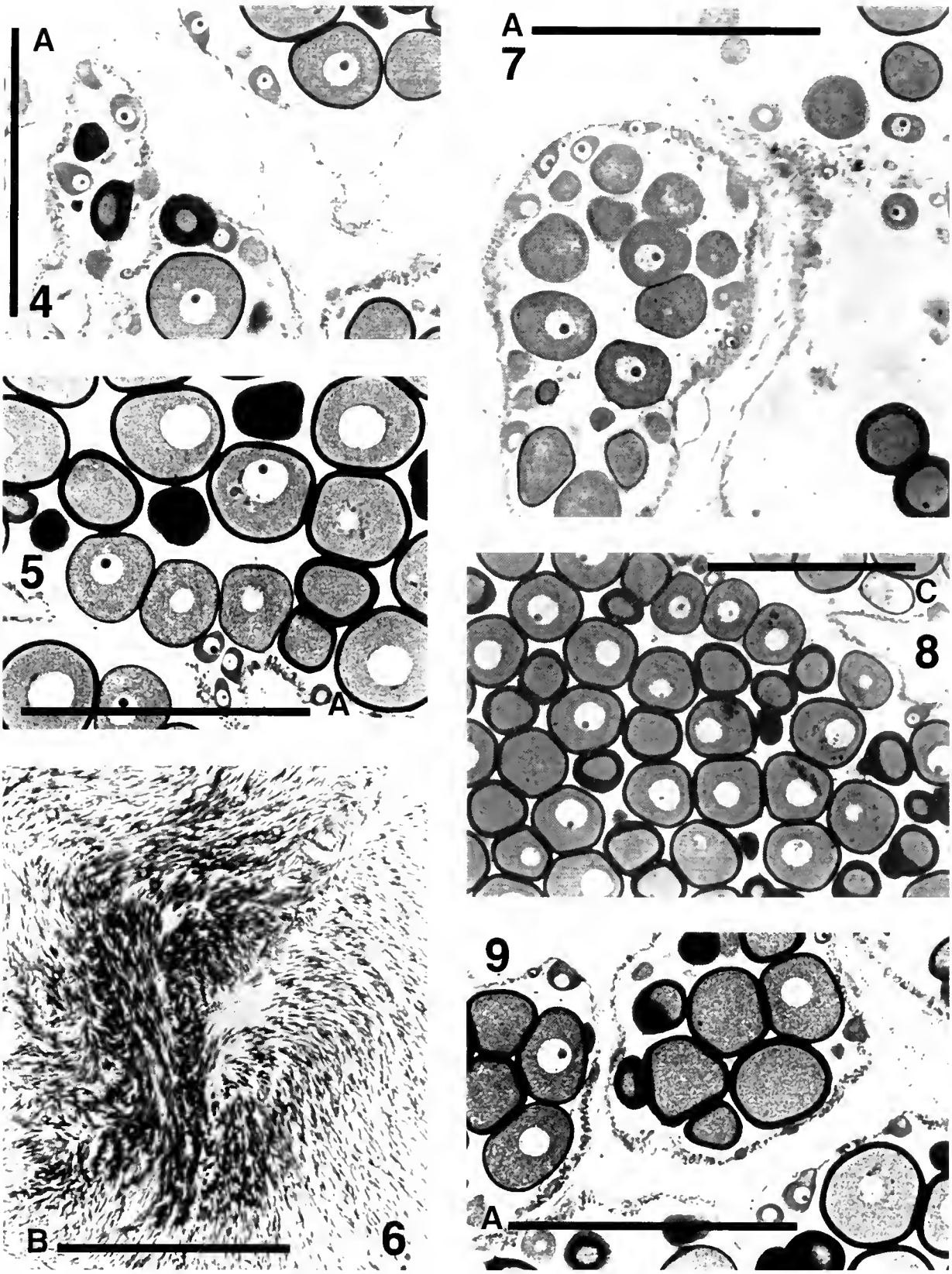

Figures 4–9. Light micrographs of *Laternula elliptica* gonad. (4) Female follicles containing both mature and new growing oocytes; (5) Most oocytes show the characteristic gelatinous layer; (6) Detail of a male follicle full of sperm cells; (7) An ovarian follicle partially spawned and another with growing oocytes; (8) Ripe ovary with packed mature oocytes. (9) Growing female follicles with both mature and immature oocytes. Scale Bar A & C = 500 microns. Scale Bar B = 100 microns.

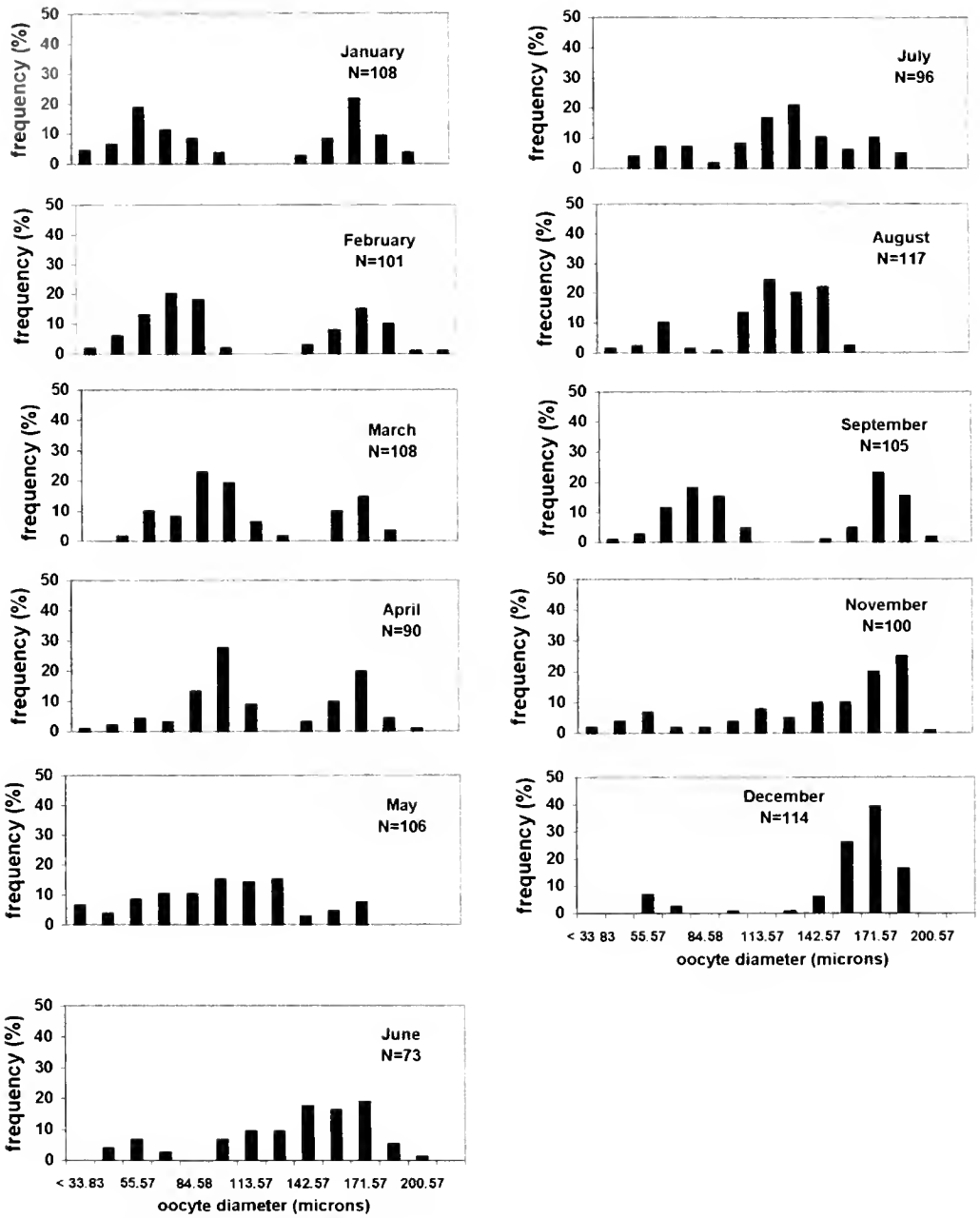

Figure 10. Frequency (%) of oocyte size (microns). N, number of measured oocytes.

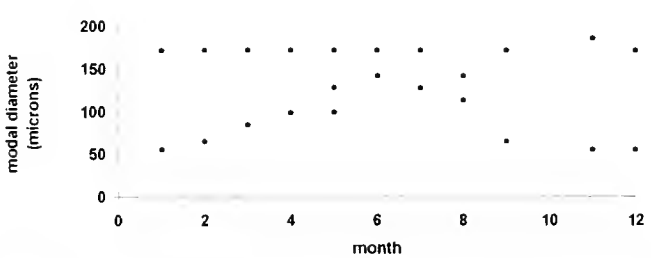

Figure 11. Modal diameter peaks of oocyte size for each sampled month in an annual cycle.

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FIRST DESCRIPTION AND SURVEY OF THE EGG MASSES OF *LOLIGO GAHI* (D'ORBIGNY, 1835) AND *LOLIGO SANPAULENSIS* (BRAKONIECKI, 1984) FROM COASTAL WATERS OF PATAGONIA

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ABSTRACT A survey was conducted on the egg masses of *Loligo gahi* (D'Orbigny, 1835), a target of an important squid fishery in the Southwest Atlantic, and *L. sanpaulensis* (Brakoniecki, 1984), a species exploited as a by-catch species in artisanal fisheries from Brazil to Argentina. The egg masses are described, and several spawning areas are identified. *L. gahi* egg masses were composed of a variable number of capsules (6–345) enclosing 40–150 eggs/embryos. *L. sanpaulensis* egg masses consisted of numerous capsules (6–465) containing approximately 240–320 eggs/embryos. The size range of the eggs was 2.1–2.3 mm for *L. gahi* and 1.2–1.3 mm for *L. sanpaulensis* from Nuevo Gulf, showing good correspondence with size of the mature oocytes carried in the oviducts by the females of each species at the same location. *L. gahi* egg masses were located in San Matías, the San José and Nuevo gulfs, Cape Tres Puntas, and Beagle Channel, usually attached to hard substrates (e.g., kelp) or objects laying on the seafloor (e.g., stones, shells, ropes, or even fishing lines) at depths from 1–15 m. *L. sanpaulensis* egg masses were found in Nuevo Gulf on sandy and muddy bottoms at depths of 5–15 m. Throughout a survey conducted from March 1996 to February 2000 in Nuevo Gulf, in an area within the distribution range of both species, *L. gahi* egg masses were found more frequently; *L. gahi* egg masses were observed every month of the year but February, whereas *L. sanpaulensis* egg masses were detected only from February to May.

KEY WORDS: *Loligo gahi, Loligo sanpaulensis*, egg masses, eggs, embryos, spawning grounds, Argentina, Patagonia

INTRODUCTION

Two loliginid squid species inhabit the Atlantic coast of Patagonia: *Loligo gahi* (D'Orbigny, 1835) and *L. sanpaulensis* (Brakoniecki, 1984). *L. gahi* is a species distributed in the southeastern Pacific from Perú to Tierra del Fuego (Cardoso et al. 1998) and in the southwestern Atlantic from Tierra del Fuego to 36°S (Castellanos & Cazzaniga 1979, Vigliano 1985). *L. sanpaulensis* is a species associated with coastal waters, distributed from San Jorge Gulf (Castellanos 1967) to Brazil (20°S) (Roper et al. 1984). Both species are present in the San Matías Gulf (Gonzalez 1999) and in the Nuevo and San Jose gulfs (Ré et al., unpublished manuscript). The fishery of *L. gahi* in the southwestern Atlantic is the second most important loliginid fishery worldwide, reaching average annual captures over 56,000 t during 1988–1997 (FAO 1999). *L. sanpaulensis* constitutes a by-catch species for local fisheries along the Atlantic coast of South America from 23°S (Costa & Fernandes 1993) to 43°S (Castellanos & Cazzaniga 1979).

The spawning areas of loliginid squids have been studied in some commercially valuable species (Vecchione 1988, Augustyn 1990, Baddyr 1991, Porteiro & Martins 1992, Sauer et al. 1992, Sauer et al. 1993). The data on the spawning areas of loliginids in the southwestern Atlantic are scarce (Andriguetto & Haimovici 1996, Ré et al., unpublished manuscript, Arkhipkin et al., 2000). Moreover, the location of these areas has been a matter of speculation until recently (Chesheva 1990, Hatfield et al. 1990, Andriguetto & Haimovici 1996).

The egg masses of Loligo are typical among those of the cephalopods (Arnold & Williams Arnold 1977, Sweeney et al. 1992); however, there are aspects (e.g., the number of capsules per egg mass, the number of eggs per capsule, the egg sizes, and the type of substratum on which the egg masses are attached) that are important to characterize the egg masses to the taxonomic level of species and that have not been reported in *L. sanpaulensis* and have been studied only recently in *L. gahi* (Guerra et al. 2001, Arkhipkin et al. 2000). For both species, the existence of one or more laying periods at different locations is still a subject of debate (Hatfield et al. 1990, Hatfield 1991, Costa & Fernandes 1993, Andriguetto & Haimovici 1996). The aims of the present study were (1) to find and identify the egg masses of *L. gahi* and *L. sanpaulensis*, (2) to locate spawning areas of both species along the Atlantic coast of Patagonia, (3) to study the seasonality of both species' spawning in a given area, and (4) to relate seasonality to the local temperature regime to identify critical values of temperature that could restrict or favor spawning.

MATERIALS AND METHODS

A continuous survey on the egg masses deposited by female loliginid squids in Nuevo Gulf, Argentine Patagonia (Fig. 1), was conducted between March 1996 and June 1998. Four buoyed ropes with weights attached were deployed on the sea bottom at Ameghino Point (March 1996-June 1996) and Kaiser Beach (July 1996-June 1998) (Fig. 1) at depths from 5–15 m (200—1,000 m from the coast). To stimulate *Loligo* females to lay their eggs on the ropes, artificial egg masses made up of small polyethylene bags filled with polystyrene spheres were attached to the ropes' bottom ends. The ropes were monitored every 2–4 wk depending on weather conditions. Seawater temperature, salinity, and pH were registered with a manual electronic sensor (Horiba Inc., Kyoto, Japan) at every control date. From August 1996 to February 2000, eight other spawning sites were found at Nuevo and San José gulfs and Beagle Channel (Fig. 1) by scuba diving and by collection of stranded egg masses on the beach. Additionally, two Loligo spawning sites were identified at Cape Tres Puntas and San Matías Gulf (Fig. 1) from the analysis of egg masses provided by Dr. Alejandro Petovello (Santa Cruz Province Fisheries Department) and Dr. Raúl Gonzalez (Almirante Storni Marine Biology Institute).

The capsules from each egg mass were counted, and the average number of eggs/embryos per capsule was estimated from a 10-capsule sample per egg mass or from the capsules available when less than 10. The embryonic stages present in each egg mass

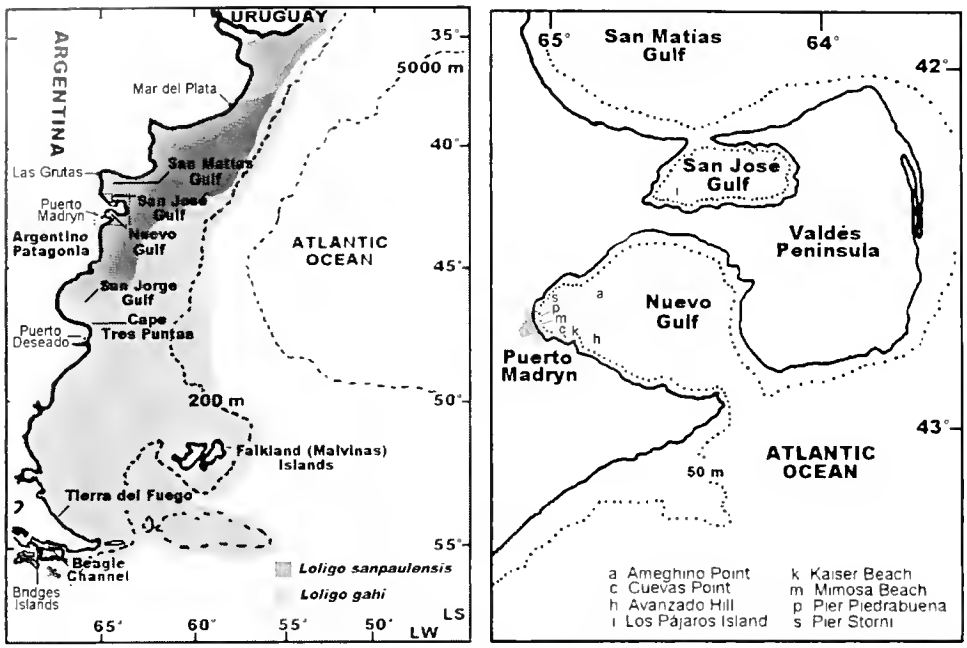

Figure 1. Area of distribution of *Loligo gahi* and *L. sanpaulensis* in Patagonia and location of the spawning areas identified in this study. Data from Vigliano (1985), Inada et al. (1986), and personal observations.

were identified following Arnold's scale (Arnold 1965). A 100-egg sample per egg mass (10 eggs × 10 capsules) was measured in egg masses showing embryonic stages previous or equal to 13 (beginning of the blastoderm expansion; Arnold 1965). On the basis of the egg counts and measurements, the egg masses were classified into different types. To identify these egg mass types to the species level, samples of the mature oocytes were taken from the ovaries and oviducts of 10 *L. gahi* (89–192 mm ML) and 10 *L. sanpaulensis* (67–157 mm ML) mature females captured by coastal seine and jigging at Nuevo Gulf. For each species, the lengths of 200 oocytes (20 oocytes × 10 females) were measured. Additionally, samples of 100 eggs/mature oocytes were randomly taken in triplicate from each of the egg mass types and from the ovaries and oviducts of the *L. gahi* and *L. sanpaulensis* mature females (3 × 10 oocytes × 10 females) from Nuevo Gulf, dried to constant weight, and weighed. All measurements were done at 25× magnification with a Wild dissecting microscope (Wild, Heerbrugg, Switzerland) equipped with an eyepiece; weights were taken to the nearest 0.01 g using a Mettler PC 440 – Delta Range electronic scale (Mettler Instruments, Zürich, Switzerland). The hatching paralarvae from each egg mass type were characterized on the basis of their mantle lengths and chromatophore arrangements.

RESULTS

Two egg mass types with distinctive eggs/embryos sizes and numbers of eggs per capsule were found in Nuevo Gulf. Each egg mass type, and also the capsules, eggs, and embryos themselves, will be hereafter referred to as type 1 and 2. The type 1 Loligo egg masses were regularly found attached to the ropes at the two locations of the same gulf where they were deployed: Ameghino Point and Kaiser Beach (Fig. 1; Table 1). They were also located by scuba diving on gravel substrate or on objects such as shells, ropes, or fishing lines at different locations of the same gulf: Pier Piedrabuena (Puerto Madryn), Cuevas Point, Avanzado Hill, and by collection of stranded material on Mimosa Beach (Fig. 1; Table 1). Type 1 egg masses were also found throughout a wide latitudinal range along the Atlantic coast of Patagonia: Las Grutas (San Matías Gulf), Los Pájaros Island (San José Gulf), Puerto Deseado (Cape Tres Puntas), and Bridges Islands (Beagle Channel). Type 2 egg masses were found only by scuba diving, anchored in sandy or muddy bottoms of Nuevo Gulf, near the pillars of Pier Almirante Storni (Puerto Madryn), and in the vicinity of Las Piedras Park, a recreational diving spot located a few hundred meters from the Puerto Madryn's waterfront (Fig. 1; Table 1).

Type 1 and 2 egg masses are well-defined structures and are made up of several capsules attached to each other and to the substrata at their basal ends by short stalks. Each capsule consists of an inner core of jelly surrounded by a spiral band of jelly that contains a row of eggs and an external covering formed by several layers of translucent material that provides cohesion and protection to the eggs (Fig. 2). The bases of the stalks, composed of the same material as the capsule's core and deprived of any external layer, are entangled, forming a bundle that is attached to the substrate. The number of capsules per egg mass varied from less than 10 to more than 300 for the type 1 egg masses and from less than 10 to more than 400 for the type 2 egg masses (Table 1). Some of the egg masses found during the survey consisted of groups of capsules, each one containing embryos in a particular stage of development (multiple egg masses; Table 1). This can be attributed to the deposition of egg capsules in a common egg mass by more than one female at various time intervals. The average number of eggs/embryos per capsule was 69 (n = 340) for the type 1 and 298 (n = 50) for the type 2 egg masses, but these numbers varied considerably between egg masses of the same type (Table 1).

Both type 1 and 2 eggs were approximately oval in shape, with the animal pole more pointed than the vegetal pole. The average size of the eggs was 2.2 mm (2.1–2.3 mm; n = 500) for type 1 egg masses and 1.2 mm (1.2–1.3 mm; n = 200) for type 2 egg masses (Fig. 2). The dry weights of 100 eggs were 0.10 g for each of the three replicates of type 1 eggs and 0.03 g for each of the three replicates of type 2 eggs.

TABLE 1.

Results of the survey on Loligo spp. egg masses in the Atlantic coast of Patagonia.

Record	Location	Date of collection	Depth (m)	pH	Temperature (°C)	Salinity (ppt)	Capsules per egg mass	Average eggs per capsule	Stage of embryonic development	Type
1*	Las Grutas	5/11/84	5	n/a	n/a	n/a	15	52	13–22	1
2	Puerto Deseado	12/15/95	Stranded	n/a	n/a	n/a	n/a	55	28	1
3	Ameghino Point	3/12/96	10	8.1	16.3	34.6	65	46	28	1
4	Ameghino Point	3/12/96	5	8.1	16.3	34.6	6	52	16	1
5	Ameghino Point	3/28/96	10	8.08	16.3	34.6	20	80	28	1
6	Ameghino Point	4/26/96	10	8.18	14.5	35.0	10	63	29	1
7	Los Pájaros Island	5/5/96	Stranded	n/a	15	n/a	153	92	17	1
8	Ameghino Point	5/29/96	15	8.2	12.9	35.0	35	65	29	1
9*	Ameghino Point	5/29/96	10	8.2	12.9	35.0	37	94	18–23	1
10	Ameghino Point	5/29/96	10	8.2	12.9	35.0	22	63	12	1
11	Kaiser Beach	7/26/96	15	8.21	10.6	35.1	78	61	10	1
12	Pier Piedrabuena	8/13/96	5	8.22	10.2	35.1	38	53	15	1
13	Kaiser Beach	8/29/96	15	8.26	10.6	35.2	52	58	15–17	1
14	Kaiser Beach	9/12/96	10	8.3	11	34.8	49	81	14	1
15	Kaiser Beach	9/12/96	5	8.31	10.8	35.1	123	69	13	1
16	Kaiser Beach	9/12/96	10	8.3	11	34.8	23	63	13	1
17*	Kaiser Beach	9/30/96	10	8.29	11.8	35.1	345	63	12-21-30	1
18	Kaiser Beach	10/14/96	10	8.3	13.2	34.6	85	85	19	1
19	Kaiser Beach	10/14/96	10	8.3	13.2	34.6	98	42	13–18	1
20*	Kaiser Beach	11/27/96	5	8.4	15.4	34.7	69	44	28–29	1
21	Kaiser Beach	11/27/96	5	8.4	15.4	34.7	30	76	10	1
22	Kaiser Beach	12/26/96	5	8.35	14.9	34.6	13	39	0	1
23	Kaiser Beach	1/28/97	10	8.46	18	34.8	85	73	28	1
24	Kaiser Beach	1/29/97	10	8.46	18	34.8	63	82	13	1
25	Kaiser Beach	3/4/97	10	8.44	19.3	34.2	7	91	8	1
26	Kaiser Beach	3/25/97	5	8.49	16.5	34.8	3	63	29	1
27	Cuevas Point	6/23/97	5	8.58	12.8	35.0	58	74	26	1
28	Cuevas Point	6/23/97	5	8.58	12.8	35.0	60	82	19	1
29	Cuevas Point	6/23/97	5	8.58	12.8	35.0	24	88	10	1
30	Mimosa Beach	7/3/97	Stranded	n/a	n/a	n/a	62	55	29	1
31	Kaiser Beach	9/10/97	10	n/a	n/a	n/a	243	48	n/a	1
32	Bridges Islands	6/15/97	12	n/a	n/a	n/a	51	74	12	1
33*	Pier Storni	3/17/98	15	8.5	16.8	34.2	180	309	Various	2
34	Pier Storni	3/17/98	15	8.5	16.8	34.2	465	322	Various	2
35	Pier Storni	3/26/98	15	8.4	16.5	34.0	13	243	13	2
36*	Pier Storni	3/26/98	15	8.4	16.5	34.0	82	314	Various	2
37	Avanzado Hill	3/31/98	1	n/a	n/a	n/a	28	154	11	1
38	Pier Storni	4/4/98	15	8.22	16.3	34.0	88	n/a	14-22-26	2
39	Pier Storni	4/4/98	15	8.2	16.3	34.0	52	n/a	29	2
40	Pier Storni	5/22/98	15	8.07	14.8	34.0	6	n/a	>29	2
41	Kaiser Beach	6/30/98	10	8.25	11.2	34.3	138	60	12	1
42*	Bridges Islands	8/17/98	6	n/a	5	n/a	35	63	Various	1
43	Las Piedras Park	2/4/00	5	n/a	17.4	n/a	20	302	21	2

No egg masses were found on the ropes on 8/13/96, 1/18/97, 2/17/97, and 4/23/97. Embryonic stages follows the scale of Arnold (1965).
n/a: not available.
*Multiple egg mass (groups of capsules with embryos at different stages of development).

Besides larger absolute sizes, type 1 embryos show external yolk sacs proportionally larger than those of type 2 embryos at the same stages of development (Fig. 3). Therefore, throughout their development, type 1 embryos show features closer to those described by Naef (1928) for the embryos of *L. vulgaris* (Lamark, 1798), and type 2 embryos show characteristics that resemble those illustrated by Arnold (1965) for the embryos of *L. pealei* (Le Sueur, 1821). At hatching, type 1 embryos attain mantle lengths of 2.6–3.2 mm, and type 2 embryos attain mantle lengths of 1.4–1.7 mm (Fig. 3). The most common chromatophore arrangements found on the embryos of both types of paralarvae are shown in Figure 4. Chromatophores were red or yellow on the ventral surface of both types of paralarvae, brown or yellow on the dorsal surface of type 1 paralarvae, and only yellow on the dorsal surface of the type 2 paralarvae. Orange chromatophores were observed only on the embryos in stages 26–28 of Arnold's scale (Arnold 1965). The cheek patches of the type 1 hatchlings consisted in four red chromatophores, but three chromatophores on either one or both cheeks were frequently observed. The type 2 hatching paralarvae displayed only two red chromatophores on each cheek patch.

The average size of the mature oocytes sampled from the ovaries and oviducts of 10 *L. gahi* females was 2.2 mm (2.0–2.4 mm; $n = 200$); the average size of the oocytes sampled from 10 female

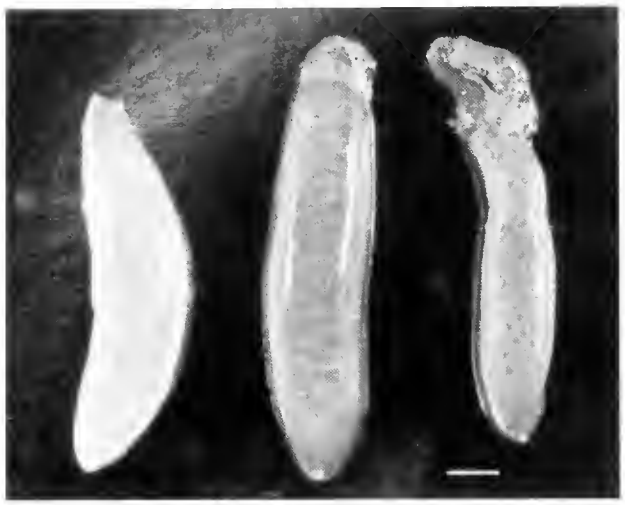

Figure 2. Aspect of *Loligo spp.* egg mass types found in the Atlantic coast of Patagonia. Left: type 1 egg capsule from Beagle Channel, Center: type 1 *Loligo sp.* egg capsule from Nuevo Gulf, Right: type 2 egg capsule from Nuevo Gulf (scale bar = 6 mm).

L. sanpaulensis was 1.2 (1.2–1.3 mm; $n = 200$). The dry weights of 100 oocytes were 0.10 g for each of the three replicates of *L. gahi*'s oocytes and 0.03 g for those of *L. sanpaulensis*. Given that *L. gahi* and *L. sanpaulensis* are the only loliginids that inhabit the waters of northeastern Patagonia (Castellanos & Cazzaniga 1979, Inada et al. 1986), the striking correspondence between the sizes and weights of each egg type and the mature oocytes of either species make it possible to state that the type 1 egg masses belong to *L. gahi* and type 2 egg masses belong to *L. sanpaulensis*.

From the egg masses obtained in Bridges Islands (Fig. 1; Table 1), those found in August 1998 showed eggs identical in size to the *L. gahi* eggs from Nuevo Gulf (average 2.2 mm, range 2.1–2.3 mm; $n = 200$). The egg mass found in the same locality in June 1997 (Table 1) showed eggs somehow bigger than those normally found in the type 1 egg masses (average 2.6 mm, range 2.3–3.0 mm; $n = 200$). Moreover, this was composed of two different groups of capsules showing eggs that were 2.3–2.6 mm and 2.6–3.0 mm. However, the number of eggs per capsule fell into the range reported for the type 1 egg masses (Table 1). The dry weight of each of the three 100-egg replicates taken from this egg mass was 0.20 g.

In Nuevo Gulf, *L. gahi* spawned almost continually throughout the year; February was the only month in which egg masses were not found (Table 1). According to historical registers (more than 10 years, hourly records) taken at Nuevo Gulf (unpublished data, Puerto Madryn's Tides Control Station, Hydrographic Service, Argentine Navy), the monthly average of daily minimum SST reaches a minimum in August (7.3°C), and the monthly average of daily maximum SST reaches a maximum in February (23°C). SST registered in Beagle Channel at the time of collection of type 1 egg masses (August 1998) was 5°C. These data show that *L. gahi* can spawn at temperatures as low as 5°C and that spawning could be limited at temperatures close to 23°C. Although the sampling procedure limits the quantitative estimation on spawning peaks of *L. gahi* in Nuevo Gulf, it is interesting that *L. gahi* egg masses with higher numbers of capsules were registered in September in both 1996 and 1997 (Table 1). Type 2 egg masses were found in Nuevo Gulf from March 1998 to May 1998 and in February 2000 (Table 1). The analysis of the embryonic stages found in these egg masses

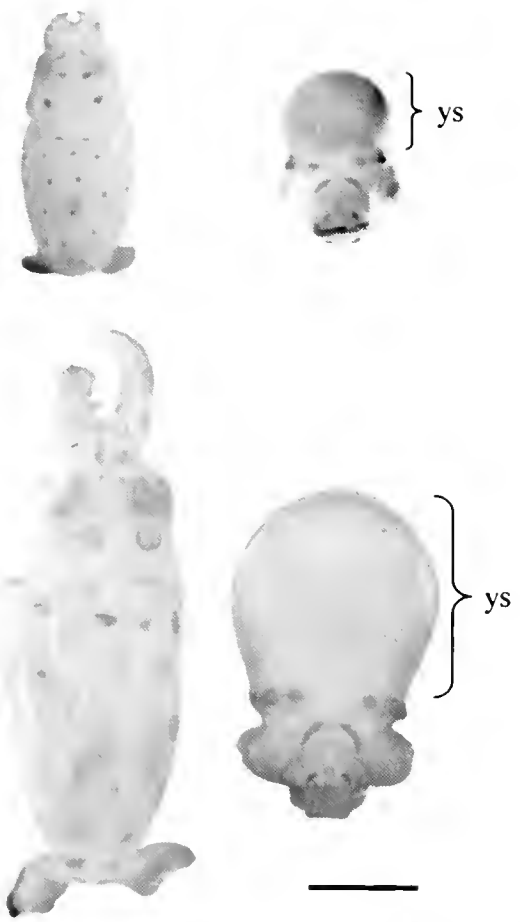

Figure 3. Compared aspect of the type 1 and type 2 *Loligo* fixed embryos. Upper left: type 2 embryo at stage 30; upper rigth: type 2 embryo at stage 21; lower left: type 1 embryo at stage 30; lower rigth: type 1 embryo at stage 21 (scale bar = 1 mm). ys: yolk sac. Embryonic stages following the scale of embryonic development of Arnold (1965).

suggests that the egg masses were actually deposited from March 1998 to April 1998 and in January 2000. These results show that the spawning season of *L. sanpaulensis* in the study area extends from summer to early fall. During this period, the monthly average of daily minimum SST reaches a minimum in April (12°C), and the monthly average of daily mean SST ranges between 15°C (April) and 17.5°C (February).

DISCUSSION

It has been observed that some loliginids deposit their egg masses on the ropes and PVC pipes used as traps for octopuses (Porteiro & Martins 1992, Ré et al. 1996). Also, artificial egg masses such as those used in this work have been reported to cause a visual stimulus for loliginids to mate and spawn (Arnold & Williams Arnold 1977, Yang et al. 1986, Vecchione 1988). The devices deployed in this study to examine the laying activities of the Loligo species present in Nuevo Gulf are a simplification of those traps. These structures stimulated the spawning of *L. gahi* but did not show any results with *L. sanpaulensis*. This seems reasonable, considering that *L. gahi* deposits its egg masses on hard substrata and *L. sanpaulensis* spawns on soft bottoms. A distinct selection of substrata for egg laying has also been observed in two sympatric loliginids in the Gulf of Mexico, *L. pealei* selecting hard substrates and *L. plei* (Blainville, 1823) preferring soft

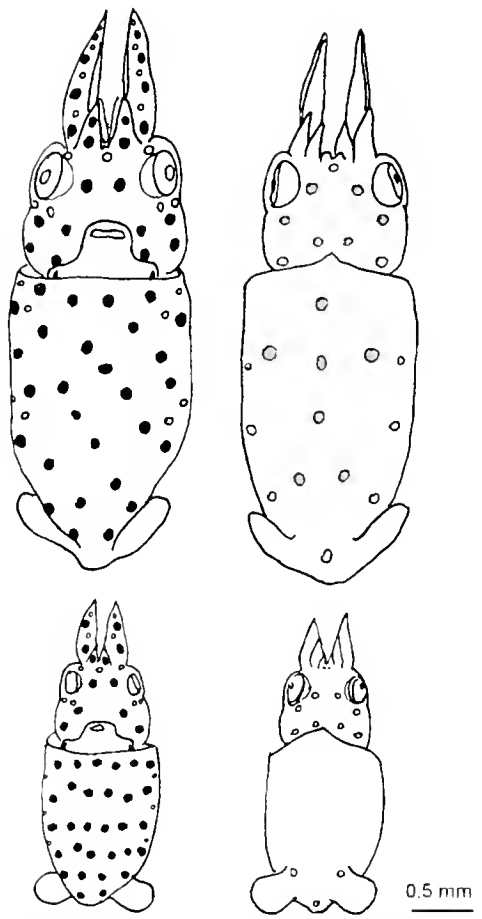

Figure 4. Chromatophore arrangements on the ventral (left) and dorsal (rigth) surfaces of the *Loligo spp.* hatching paralarvae from coastal waters of Patagonia. Top: type 1 paralarva, bottom: type 2 paralarva. Black circles represent red chromatophores, gray circles represent brown chromatophores and empty circles represent yellow chromatophores.

substrates (Vecchione 1988). Arkhipkin et al. (2000) found egg masses of *L. gahi* attached to no other substrate than kelp and concluded that the spawning sites of the species could be limited to shallow waters. In the present work, egg masses of *L. gahi* have been found attached not only to kelp but also to other types of hard substrate.

The structure of both species' egg masses, several capsules enclosing a row of eggs arranged in a coiled pattern along their longitudinal axle, is typical of Loligo species (Arnold & Williams Arnold 1977). The number of capsules per egg mass is variable for *L. gahi* and *L. sanpaulensis* egg masses; this variability is probably related to the size and physiological condition of the mother. This has also been observed in *L. vulgaris* reynaudii (Lamark, 1798) by Sauer et al. (1993), who found concentrations of egg masses more than 3 m in diameter surrounded by smaller ones (1–10 capsules per egg mass), and by Vecchione (1988), who observed Loligo sp. egg masses in the Gulf of Mexico that were made up of 10–40 capsules. However, as it has been observed in the present study, the variability could in part be the result of the deposition of multiple egg masses on a common mass by more than one female. Also, it should be noted that even when most egg masses were considered as individual egg masses, it is possible that these were deposited by more than one female within a brief period of time, so they could not be distinguished from each other on the basis of embryo maturity. This has also been suggested by Arkhipkin et al. (2000) who found 6–141 capsules per egg mass in *L. gahi*.

The number of eggs per capsule in the egg masses of *L. gahi* is comparable to that reported for other loliginids, such as *L. duvaucelii* (D'Orbigny, 1848) (125–150 eggs per capsule; Asokan & Kakati 1991), *L. forbesi* (Steenstrup, 1856) (39–52 eggs per capsule; Porteiro & Martins 1992), and *L. vulgaris reynaudii* (148 ± 37 [mean ± SD] eggs per capsule; Sauer et al. 1993). The number of eggs per capsule reported in this study is comparable to that found by Guerra et al. (2001) (50–60 eggs per capsule) from a 12-capsule egg mass of *L. gahi* found in Reñaca, Chile (32°58′S, 71°32′W). Also, the mean number of eggs per capsule observed in the present work (69 eggs per capsule) is similar to that reported by Arkhipkin et al. (2000) for the same species (71 eggs per capsule). The number of eggs per capsule of *L. sanpaulensis* is comparable to that for *L. plei* (200–300 eggs per capsule) reported by Waller and Wicklung (1968).

The egg-size range of *L. opalescens* (Berry, 1911) (2.0–2.5 mm; Fields 1965) shows the greatest resemblance to that of *L. gahi*. Other species with close egg-size ranges are *L. vulgaris* (2.3–2.7 mm) (Worms 1983, cited in Baeg et al. 1992) and *L. bleekeri* (Keferstein, 1866) (2.6–2.7 mm; Baeg et al. 1992). The size range observed in the eggs of *L. gahi* from Nuevo Gulf and the egg mass found in Bridges Islands in August 1998 (2.1–2.3 mm) is within the range reported by Arkhipkin et al. (2000) (2.0–2.5 mm) from egg masses found in the Falkland (Malvinas) Islands, and the size range from the eggs found in Bridges Islands in June 1997 (2.3–3.0 mm) is comparable to that reported by Guerra et al. (2001) from the eggs found in Reñaca, Chile (2.6–3.1 mm). On the other hand, the size range of the *L. sanpaulensis* eggs falls within the range reported for the Northeastern Atlantic species *L. pealei* (1.1–1.6 mm; Summers 1983, cited in Baeg et al. 1992).

The chromatophore arrangements displayed by the hatching paralarvae of *L. gahi* and *L. sanpaulensis* were easily distinguishable from each other. The pattern found in *L. gahi* closely resembles that reported by McConathy et al. (1980) for *L. opalescens*, and the pattern found in *L. sanpaulensis* is similar to that observed by the same authors in *Lolliguncula brevis* (Blainville, 1823). The pattern of chromatophore arrangements reported by Arkhipkin et al. (2000) for the paralarvae of *L. gahi* is similar to that found in this study. However, this authors omitted two yellow chromatophores placed on the ventral surface of the head, anterior to the eyes, that were regularly observed in the *L. gahi* hatchlings from this study, both from Nuevo Gulf and Bridges Islands, and in other loliginids (Naef 1928, McConathy et al. 1980, Baeg et al. 1992, Blackburn et al. 1998). Also, Arkhipkin et al. (2000) incorrectly report the presence of brown chromatophores on the ventral surface of the hatching paralarvae, which are actually red, and the presence of orange chromatophores, which are actually yellow (orange chromatophores are present only in earlier stages of the embryonic development).

L. gahi shows a year-round spawning season in Nuevo Gulf, which could be limited at the depth range covered in this study (0–15 m) by the maximum SST of the hottest month. Continuous spawning throughout the year has also been reported for *L. vulgaris* reynaudii (Sauer et al. 1992) from South Africa and *L. forbesi* (Lum Kong et al. 1992) from Irish waters. The observation of two peaks of recruitment of *L. gahi* to the Falkland (Malvinas) Islands fishery has led some authors to consider two major spawning peaks for that area (Patterson 1988, Hatfield et al. 1990).

Recently, a third recruitment peak has been identified (Agnew et al. 1998, Hatfield & Murray 1999). The hypothesis of a spawning peak in September for *L. gahi* in Nuevo Gulf, on the basis of this study's preliminary observations on the size of the egg masses, agrees with the observations of Portela et al. (1994), who reported maximum proportions of spawning *L. gahi* individuals in September between 42°S and 49°S during a survey conducted from March to October 1989.

On the basis of the results of the egg mass survey in Nuevo Gulf, it can be stated that the spawning activity of *L. sanpaulensis* near the southern limit of its geographic distribution is restricted to summer. Unpublished data on the reproductive cycle of *L. sanpaulensis* in Northern Patagonia on the size and maturity structure of the adult and juvenile population (Barón & Ré, Pers. Comm.) is consistent with this conclusion. Previous studies on the reproductive cycle of *L. sanpaulensis* in fishing areas of central Argentina (Mar del Plata, 38°S) and Brazil (Cabo Frio, 21°S) indicated two spawning peaks per year (Vigliano 1985, Costa & Fernandes 1993). However, an extended spawning period for *L. sanpaulensis* throughout the year has been considered for southern Brazil in a more recent study (Andriguetto & Haimovici 1996). In Nuevo Gulf, spawning seems to be limited by water temperature below 12°C and facilitated by a monthly average of daily mean temperature in the range of 15–17.5°C. This temperature range is in agreement with the observations of Andriguetto and Haimovici (1996) who reported the finding of undetermined Loligo egg masses, presumably of *L. sanpaulensis*, in Southern Brazil at bottom temperatures of 17.5°C–17.8°C.

The differences in the sizes and weights of the eggs in the egg mass found in Bridges Islands in July 1997 cannot be fully explained on the basis of the present information. The existence of two groups of capsules enclosing eggs with different size ranges but the same stage of development suggests that this was actually a multiple egg mass deposited by two females within a brief time interval. Given that the number of eggs per capsule of this egg mass is within the range observed for type 1 egg masses, this could be a *L. gahi* egg mass adapted to the low temperature conditions of the Beagle Channel. On the other hand, it could be thought of as the egg mass of another Loligo species. Filippova (1969, cited in Castellanos & Cazzaniga 1979) postulated the existence of two different taxonomic entities within *L. gahi* (*L. gahi* in waters of the Pacific and *L. patagonica* in the Atlantic). However, Castellanos and Cazzaniga (1979) and Brakoniecki (1984) presented evidence that *L. patagonica* is a junior synonym of *L. gahi*.

The present work shows the wide ranges of latitude and temperature suitable for the spawning activity of *L. gahi* along the Patagonian Coast and the narrow range of temperature at which *L. sanpaulensis* can reproduce near the southern limit of its distribution. Further studies on the spawning grounds of *L. gahi* and *L. sanpaulensis* must emphasize quantitative estimations to provide predictive data on the recruitment patterns of both species.

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DIGESTION OF CELLULOSE BY STOMACH HOMOGENATES OF GREEN ABALONE (*HALIOTIS FULGENS*)

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ABSTRACT Three experiments were conducted to study the cellulase activity of stomach homogenates of green abalone, with and without antibiotics added. Forty-eight previously frozen stomachs from wild abalone adults were thawed and individually homogenized. Four cultured abalone adults maintained under a balanced diet containing cellulose were killed and the fresh stomach samples processed. Cellulase activity was estimated through the production of glucose, incubating the homogenate from each organism with cellulose. To determine the effect of bacteria, each stomach was incubated (at 25°C for 20 h) in a completely randomized design, with and without an antibiotic mixture. For a time series measurement (exp. 2), four homogenates from wild abalone were used; the incubation mixture was followed up taking samples at 0, 1, 2, 4, 8, 16, 20, 32, 64 and 72 h. The latter samples were also used to measure the enzymatic activity before and after a 72-h incubation, in the absence of antibiotics (exp. 3). Initial bacterial counts in the wild abalone homogenates were 459 colonies per mL. In the presence of the antibiotic mixture, no bacterial growth was observed. Initial cellulase activity was 1.6 Units. Cellulose degradation figures were low, even in the absence of antibiotics, indicating the low dependence of abalone on the nutrients derived from the digestion of the cell walls. Still, the addition of antibiotics depressed the digestion of cellulose (14.5 *vs.* 10.2%), suggesting the importance of live bacteria in the digestion process, and that the majority of the degraded cellulose is perhaps acted upon by endogenous enzymes. The time series experiment of samples from wild abalone, with and without antibiotics, showed quadratic effects on cellulose digestion $y = 7.55 + .29h - .005\ h^2$ ($r^2 = .2212$), but no significant differences between treatments. The gradual increase in the digestion of cellulose, followed by a plateau is similar to what happens in ruminant digestion. The initial and final enzyme activity without antibiotics showed a significant reduction of 41.1% for the wild abalone whereas in cultured abalone a 50.9% significant reduction was observed. It seems that the cellulase complex enzymes present in the homogenates remain active after 72 h of incubation, even though their activity is lowered by about one half. Although the use of antibiotics assure the absence of live bacteria in the incubation mixture, the cellular enzymes possibly freed by the effect of the antibiotics could still be active for up to 72 h after the microbes are no longer viable. Thus the enzymatic activity attributed to the stomach itself could still be confounded.

KEY WORDS: green abalone, *Haliotis fulgens*, cellulose, digestion, bacteria, antibiotics

INTRODUCTION

The information on the nutritional physiology of the abalone, specifically in relation to their capability to digest and metabolize polysaccharides such as cellulose, is scarce. Uki et al. (1985) and Uki and Watanabe (1992) established that abalone had a limited capacity to digest cellulose as their growth rate was inversely related to increased levels of dietary cellulose (from 0 to 20%). However, it has been observed that abalone grown in banks with predominant sea grass, with higher cellulose content, appear to be bigger and healthier than those grown in areas were kelp is the predominant vegetation. Monje and Viana (1998) compared two purified diets, one containing 19% cellulose and 1% sodium alginate and a second one in which cellulose was substituted with sodium alginate. Although both diets resulted in similar growth rates, the group fed the cellulose-based diet had higher cellulase and alginase activities than those fed the sodium alginate.

Leighton (1968) stated that the enzymes involved in the digestion of complex carbohydrates were secreted in the hepatopancreas. In contrast, Erasmus et al. (1997) suggested that bacteria might play a role in the digestive process of the gastropod, since several microbial species with cellulase activity could be detected in the digestive tract of the abalone (*H. midae*); however they registered cellulase activity even in gnotobiotic organisms.

The aim of the present experiments was to study the cellulase activity of stomach homogenates of abalone, with and without antibiotics added, using a crystalline form of cellulose as substrate.

MATERIALS AND METHODS

Forty-eight stomachs were obtained from wild abalone adults, harvested near the "Emancipación" Fishery Cooperative in Baja California. The stomachs were placed in layers on ice, and frozen at −75°C until used. They were later thawed slowly in temperate water, their pH and ion strength measured, and then individually homogenized.

Four cultured abalone adults that had been maintained under a balanced diet (Table 1) containing 5% cellulose were killed and the stomach samples immediately processed as mentioned above.

Cellulase activity was estimated using the methodology described by Worthington (1988). Where an aliquot from the stomach homogenate from each organism was incubated with 300 mg α-cellulose in the presence of a .05 M phosphate buffer solution at pH 5.5, and 37°C. After 2 h, the reaction was colored with an anthrone-H_2SO_4 solution and the production of glucose measured by spectrophotometer (Genesis 2000) at 625 nm. One unit releases 0.01 mg glucose per hour from microcrystalline cellulose at 37°C, pH 5.5, per mg of stomach homogenate (wet weight).

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TABLE 1.

Ingredient composition of the artificial diet used for the culture abalone, given as g kg^{-1} of dry matter.

Ingredients	g/kg of dry matter
Fish meal[1]	300
Fish silage[2]	50
Soy bean meal	80
Corn meal[3]	100
Kelp meal	100
Starch (rice)	164
Cellulose[4]	80
Gelatin (50 Blooms)	60
Vitamin mixture[5]	15
Mineral mixture[5]	40
Stay C[5]	2
Choline chloride	1
Methionine	2
Sodium benzoate	2
BHT[6]	4

[1] 60% protein kindly supplied by Productos de Ensenada, México.
[2] Made from tuna-fish viscera, given as dry matter basis.
[3] Maseca, produced in Mexico.
[4] Alphacel, α-cellulose.
[5] As recommended by Uki and Watanabe (1992), kindly supplied by Roche, Mexico.
[6] Butylated Hydroxytoluene.

To determine the effect of the bacteria present in the homogenates, each stomach was incubated (at 25°C for 20 h) in a completely randomized design, with and without an antibiotic mixture as recommended by Erasmus (1996): chloramphenicol (250 μg/mL), cephalosporin (250 μg/mL) and ampicillin (600 μg/mL). At the end of the incubation a 1-mL sample was taken and after centrifugation (1880 g) the supernatant was used to determine glucose through the procedure indicated previously. The effect of the presence of the antibiotic mixture was assessed using the following calculation:

Relative digestibility reduction = [(dig. a − dig. n/a) · 100]/ dig. n/a

where: (dig. a) is the digestibility with antibiotics and (dig. n/a) the digestibility without antibiotics.

The number of bacterial colonies with and without antibiotics was determined before and after incubation of the stomach sample. Thus, a Zobell media was used by blending agar (1.5 g/100 mL), peptone (.5 g/100 mL), yeast extract (.1 g/100mL) and α-cellulose .2g/100 mL, mixed with filtered seawater to distilled water at a ratio of 1:3 v/v at pH 5.5. The Petri dishes were incubated at 25°C for up to two days, with and without antibiotics in the same proportion as indicated previously. Cellulase activity from the growing bacteria was corroborated using the Congo red procedure (Teather and Wood 1982).

For a time series measurement (experiment 2), four homogenates from wild abalone stomachs were used; the incubation mixture was followed up taking samples at 0, 1, 2, 4, 8, 16, 20, 32, 64 and 72 h. Glucose was determined as indicated previously and the glucose produced over time was related to the cellulase activity.

The latter samples were used to measure the enzymatic activity before and after a 72-h incubation, in the absence of antibiotics (experiment 3).

Statistical Analysis

A one-way ANOVA test was used to compare the relative digestibility reduction in stomach contents with and without antibiotics and to compare the initial and final enzyme activity in cultured and wild abalone without antibiotics after 72 h of incubation. Figures given in percentage were transformed to their arcsine sqr (y) to be analyzed. For the time series a quadratic and linear regression model was used to compare the slopes with a variance analysis to estimate the best adjustment according to Zar (1999). The SAS program was used to analyze the data.

RESULTS

The initial bacterial counts in the wild abalone homogenates were 459 colonies per mL of stomach. In the presence of the antibiotic mixture, no bacterial growth was observed. The initial cellulase activity of wild abalone homogenates was 1.6 Units. The *in vitro* digestibility of cellulose after 20 h incubation was lower when the antibiotic mixture was present (14.5 *vs.* 10.2%, respectively) although the difference was not significant ($P = .107$; $F = 2.666$). The relative digestibility reduction was 29.7% (Table 2).

The time series experiment of samples from wild abalone, with and without antibiotics (Table 3), showed quadratic effects on the digestion of cellulose $y = 7.55 + .29h - .005h^2$ ($r^2 = .2212$), but no significant differences between treatments. The estimated equations were $y = 8.35 + .31h - .005h^2$ for the treatment without antibiotics and $y = 6.76 + .29h - .004h^2$ for the treatment with antibiotics; the statistical analysis of these equations showed similar slopes.

For the cultured abalone, with and without antibiotics, there was also a quadratic effect, with significant differences ($P < .05$) between 4 and 16 h.

The initial and final enzyme activity without antibiotics showed a reduction of 41.1% ($P = .032$) for the wild abalone whereas in the cultured abalone a 50.9% ($P = .046$) reduction was observed (Table 4).

DISCUSSION

Mammalian cellulose-digesting herbivores can be divided into two groups: those who depend heavily on the resulting metabolites, and the ones in which the process only supplements their

TABLE 2.

Digestion of cellulose in wild abalone stomach homogenates, with or without antibiotics.

Parameter	Mean (±SD)	*n*
Total bacteria count/mL of stomach	459 (±497)	27
Initial cellulase activity	1.6 (±1.7)[1]	27
Digestibility without antibiotics, %	14.5 (±13.6)[2]	38
Digestibility with antibiotics, %	10.2 (±8.9)[2]	38
Relative digestibility reduction, %	29.7[3]	38
Significant differences between treatments	p = 0.107	

[1] mg glucose/mg stomach homogenate incubated for 2 hr at 37°C.
[2] mg glucose/mg stomach homogenate incubated for 20 hr at 25°C.
[3] Relative decrease in digestibility due to the use of the antibiotic mixture.

TABLE 3.

Digestibility of cellulose by wild abalone stomach homogenates as a function of time, with and without antibiotics. Activity is measured as the increment of glucose production. SD is given in parentheses; $n = 4$.

	Hours								
Antibiotics*	**1**	**2**	**4**	**8**	**16**	**20**	**32**	**64**	**72**
Without	4.1	8.8	10.9	14.8	12.3	12.3	11.2	4.0	3.9
	(1.1)	(2.2)	(2.5)	(3.6)	(3.8)	(3.8)	(3.6)	(2.6)	(2.2)
With	4.7	8.1	8.3	11.0	8.9	10.5	12.1	4.3	4.9
	(1.0)	(1.4)	(1.6)	(3.5)	(1.7)	(3.5)	(2.9)	(2.2)	(1.6)

* A mixture of chloramphenicol (250 μg/mL), cephalosporin (250 μg/mL) and ampicillin (600 μg/mL) as recommended by Erasmus (1996) was used.

nutritional budget. Still, both groups are unable to degrade cellulose with their own enzymes, relying instead on symbiotic microorganisms to perform the process. In any case, the digestibility of cellulose, which varies according to a number of factors (Merchen and Bourquin 1994), can be expected to be in the 40-60% and the 0-20% ranges for both animal groups, respectively (Van Soest 1994).

Symbiotic bacteria of mammalians' guts digest cellulose by attaching themselves to the substrate and then liberating the respective enzymes, which are diffused into the complex structural matrix of cellulose. These enzymes are produced either extracellularly or bound in the membrane of microbes. However, when the microbial cells are destroyed, the freed enzymes might still act directly on the cellulose molecules. For example, the ampicillins and cephalosporins used here, act on the bacteria by the activation of enzymes that destroy the cell wall, whereas chloramphenicol acts inhibiting bacterial protein synthesis.

In the experiment reported here, cellulose degradation figures were somehow low, even in the absence of antibiotics, which could be an indication of the low dependence of abalone on the nutrients derived from the digestion of the cell wall component. Still, the addition of the antibiotic mixture depressed the disappearance of cellulose by around one third, suggesting the importance of live bacteria in the digestion process, and that the majority of the degraded cellulose is perhaps acted upon by endogenous enzymes.

Erasmus et al. (1997) obtained bacteria-free abalone after immersing them for 72 h in sea water containing an antibiotic mixture. Unfortunately, after several attempts to get gnotobiotic *H. fulgens* in our lab, we were not able to repeat their procedure, either by the addition of antibiotics to the water or in the food. In all cases a considerable amount of bacteria appeared in the stomachs.

Moreover, to determine cellulase activity, Erasmus et al. (1997) used carboxymethyl cellulose, a chemically modified soluble presentation of cellulose, as substrate to determine cellulase activity. It is known that any form of cellulose that is either acid or alkali-treated has little effect on the overall cellulase system for most of the known microorganisms (Worthington 1999). The enzymatic mechanism whereby certain microorganisms can quite rapidly and completely degrade cellulose is not completely understood. However, according to Worthington (1999), two steps involved in the degradation are proposed: first, a pre-hydrolytic step wherein anhydro-glucose chains are swollen or hydrated, involving an enzyme designated C_1. The second step involves the hydrolytic cleavage of the now susceptible polymers either randomly or endwise with an enzyme complex termed $C\chi$ consisting of exo and endo β-1,4 glucanases and β-glucosidase (cellobiase) that attack soluble derivatives or cellulose that has been swollen by acid or alkali. Therefore it is recommended that in order to test true cellulase activity, the most crystalline form of cellulose available be used. In the present work α-cellulose was used.

The effect observed with the homogenates from the wild organisms in the first 32 h of incubation, is similar to what might be expected to occur in the digestive tract of the large mammalian herbivores (Van Soest 1994); that is, a gradual increase in the digestion of cellulose (as the so-called digestion rate in ruminants), followed by a plateau (equivalent to what is known as the extent of the fermentation in ruminants). The low figures for the 64 and 72-h samples could indicate the destruction of the glucose formed from cellulose, due to the long incubation period.

The results of experiment 3 could be interpreted in two ways: either that the cellulase enzymes present in the stomach homogenates remain active even after 72 h of incubation, although their capability to cleave the substrate is lowered by about one half; or that the final product (*i.e.* glucose), used as indicative of the cellulase activity, was partially spent as in the case of experiment 2. If this is the case, then the activity against carboxymethil cellulose presented by Erasmus et al. (1997) in stomach content after the abalone were treated with antibiotics for 72 hours could not be regarded as indicative of endogenous enzymes participation but to a remnant activity in the stomach.

Finally, it is important to stress that although the use of the

TABLE 4.

Initial and final enzyme activities from wild and cultured abalone stomach homogenates, without antibiotics, after 72 hours of incubation.

Abalone	**Enzyme activity[1]**	**Mean (±SD)**	***n***
Wild	Initial	1.58 (±1.7)	4
	Final	0.65 (±.3)	4
	Reduction	41.1%	
		$P = 0.032$	
Cultured	Initial	1.65 (±.6)	4
	Final	0.84 (±.3)	4
	Reduction	50.9%	
		$P = 0.046$	

[1] mg glucose produced/mg stomach homogenate after 2 hr incubation at 37°C.

antibiotic mixture assured the absence of live bacteria in the incubation process, the cellular enzymes freed by the effect of the antibiotics could still be active even at 72 h after the microbes were no longer viable. Thus the enzymatic activity attributed to the stomach itself could still be confounded.

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REPRODUCTIVE CHARACTERISTICS OF THE ARCHAEOGASTROPOD *MEGATHURA CRENULATA*

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ABSTRACT A histological and histochemical study was performed on individuals of the archaeogastropod *Megathura crenulata* sampled in the field, in order to ascertain the fundamental features of reproductive biology in this species. Basic aspects addressed were gonad and gamete structure, nature of vitelline reserves, and composition of oocyte coat. Stereological counts and oocyte measurements were performed to obtain a quantitative assessment of the reproductive cycle from June 1999 to June 2000. No simultaneous hermaphrodites were observed. The gonad structure of *M. crenulata* consisted of traversing trabeculae from which gametes developed centrifugally. The gonads of both males and females were homogeneous, allowing reliable data to be obtained from a single histological sample of each individual. Mature gametes greatly dominated the profile throughout the study period; coated oocyte diameters were also very stable. These techniques, routinely applied to the study of reproductive cycles, did not allow the identification of spawning preparedness in this species. Vitelline reserves were dominated by non-staining (putatively lipid) vacuoles; no appreciable quantities of glycogen were observed. The oocyte coat was chiefly composed of acid mucopolysaccharides, conferring both mechanical and antimicrobial protection, as well as limiting egg and larval dispersal.

KEY WORDS: *Megathura crenulata*, Gastropoda, gonads, gametes, reproductive cycle, histochemistry

INTRODUCTION

Many marine natural products present biological activity in humans, and are used in medical testing or in pharmaceuticals (Munro et al. 1987, Ireland et al. 1989, Kobayashi et al. 1989, Suffness et al. 1989, Faulkner 2000). The archaeogastropod *Megathura crenulata* is a keyhole limpet native to the California Pacific Coast, with a reported range from Mendocino County (40°N; 124°W) to Isla Asuncio (27°N; 115°W). Keyhole limpet hemocyanin (KLH) of this organism is used in the treatment of certain forms of bladder cancer (Harris & Markl 1999, 2000). It is thus of considerable medical and economic interest to rear this organism for extraction and purification of the active compound. This goal requires knowledge concerning essential aspects of *M. crenulata* biology, notably feeding, growth, and reproduction.

The sparse studies on reproduction in *M. crenulata* chiefly concern modes of fertilization, which is external and involves several hormonal agents (Tyler 1939, Webber 1977). The lack of data on the reproductive biology of *M. crenulata* may be contrasted with the relatively abundant information concerning the highly-prized abalones (*Haliotis spp*), which are commercially important, edible archaeogastropods (Newman 1967, Purchon 1968, Fretter 1984, Hahn 1989). The present study documents the gonad structure, oocyte histochemistry and reproductive cycle of *M.crenulata* in its natural habitat, with a view toward the long-term objective of cultivation.

MATERIAL AND METHODS

Specimen Collection and Dissection

The animals used in the present study were obtained from June 1999 to June 2000 from the subtidal zone off Long Beach, California (33°45′N, 118°10′W). The specimens were sent by air to France, where they were dissected and fixed in the SMEL (Syndicat Mixte pour l'Equipement du Littoral) laboratory in Blainville, Normandy. Five individuals were dissected each month, except in October 1999, when no sampling was possible. In order to verify the structural homogeneity of the gonad, the entire organ was removed in certain individuals of certain months, and several regions were examined (Table 1): a median region (M), a distal extremity (DE) and a proximal extremity (PE). For samples from February, May, and June 2000, tissue pieces were obtained from labelled L, M and R regions, allowing the distinction of left (L), median (M), and right (R) regions of the gonad. The types of histological sample are summarized in Table 1 for each individual and each sampling date.

Histological Techniques

In most cases, entire gonads were fixed in aqueous Bouin's solution. In an effort to improve fixation, small pieces of the gonad were fixed in March and April; however, the resulting sections showed that this provoked extensive leakage of gametes, such that the sections could not be used for males in either month, and for females in March. From February, May, and June 2000, in which the dissections allowed the distinction of three different regions of the gonad, histological samples were directly fixed. After rinsing for a minimum of 10 h and dehydration in an ascending ethanol–Bioclear® series, the biopsies were embedded in paraffin and sectioned at 7 μm.

Several staining protocols were developed for this study, based on Martoja and Martoja-Pierson (1967), Gabe (1968) and Vacca (1985), as indicated in Table 2. A modified Masson's trichrome protocol using fast green, trioxyhematein, and acid fuschin allowed the topological study of the gonads, distinguishing connective tissue, oocyte coat, cytoplasm, nucleus, chromatin, and nucleolus. Periodic acid–Schiff reagents with positive (oyster digestive gland) and negative (amylase-digested sections of gonad) controls were used to determine the eventual presence of glycogen in the oocyte cytoplasm, as well as neutral mucopolysaccharides (NMPS) in the oocyte coat. Alcian blue was used to determine the presence of acid mucopolysaccharides (AMPS) in the oocyte coat.

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TABLE 1.

Megathura crenulata. Summary of histological sample types for males and females examined.

Date	Males	Male gonad regions	Females	Female gonad regions
06/99	–	–	1	I
	–	–	2	M,P, D
	–	–	3	I
07/99	1	I	1	I
	2	M,P, D	2	I
	3	I	–	–
08/99	1	M,P, D	1	M,P, D
	2	M,P, D	2	I
	3	I	–	–
09/99	1	I	1	M,P, D
	2	I	2	I
	–	–	3	M,P, D
10/99	–	–	–	–
11/99	1	1	1	I
	2	I	2	I
	–	–	3	M,P, D
12/99	1	1	1	I
	2	1	2	I
	–	–	3	M,P, D
01/00	1	M,P, D	1	I
	2	I	2	M,P, D
	3	I	–	–
02/00	1	R, L,M	1	R, L,M
	2	R, L,M	2	R, L,M
	–	–	3	R, L,M
03/00	–	–	–	–
04/00	–	–	1	R, L,M
05/00	1	R, L,M	1	R, L,M
	2	R, L,M	2	R, L,M
	3	R, L,M	–	–
06/00	–	–	1	R, L,M
	–	–	2	I
	–	–	3	R, L,M
	–	–	4	I

I: undetermined gonad region; M: median gonad region; P: proximal region of non-oriented gonad (organ previously removed from individual); D: distal region of non-oriented gonad; R: right region of oriented gonad; L: left region of oriented gonad.

Stereology

Stereological counts were performed in order to quantify the proportions of the different tissue types in the histological sections; variations in the proportions of the different tissue categories thus reflected variations in the different phases of reproductive activity (Weibel et al. 1966, Briarty 1975, Beninger 1987, Morvan & Ansell 1988, Pazos et al. 1996, Mayhew 2000); counts were performed on surfaces of measured area, using a 9×9 point matrix on a microscope projector.

In males, four tissue categories were identified for stereological purposes: trabecular tissue, developing gametes, mature gametes, and unoccupied space. In females, three tissue categories were identified: coated oocytes, non-coated oocytes, and trabecular tissue. Due to the loose nature of the female gonad tissue, it was impossible to determine whether observed unoccupied spaces were real or artefacts of gonad dissection. Stereolological counts were therefore performed only on areas without visible unoccupied space.

Nine counts were performed for each individual and region of the gonad, and the means (with 95% confidence intervals) of all male and female counts were plotted.

Oocyte Diameters

Oocyte diameters were measured for 30 coated oocytes for each female and for each gonad region, using a calibrated optical micrometer. In order to standardize the measurements, only oocytes in which the nucleolus was visible (approximately the center of the cell) were selected. The evolution of oocyte diameters could then be recorded throughout the year.

RESULTS

Males

Gonad Structure

The general structure of the male gonad is presented in Figure 1 area 1. The gonad was composed of connective tissue trabeculae, from which arise centrifugally the germ cells, first visible as developing aflagellate gametes, and then the mature flagellate spermatozoa. The mature spermatozoa occupied the majority of the sectional area (Fig. 1 area 1, Fig. 2).

Stereology

Testicle homogeneity was verified in 1 to 3 individuals from July, August 1999 and January, February and May 2000, using stereological counts. The results shown in Fig. 2 indicate that the male gonad was structurally homogeneous and gametogenetically synchronous. Reliable data for histological study may therefore be obtained from only one histological sample per individual.

The male gonad presented a stable histological profile throughout the study period from July 1999 to June 2000. Mature spermatozoa occupied almost the entire gonad, with mean volume fractions of 0.77 (May 2000) to 0.88 (November 1999). In comparison, developing gametes occupied a low proportion of the gonad, from 0.095 (November 1999) to 0.16 (July 1999). Trabec-

TABLE 2.

Summary of stains used, and celullar and molecular components targeted.

Stain used	Structures and molecules targeted	Cellular and tissue constituents
Acid fuschin	Cytoplasmic granules	Cytoplasmic granules
Fast green	Reticulate fibres, collagen	Connective tissue
Trioxyhematein	Nucleic acids	Nucleus, nucleolus and chromatin
PAS-alcian blue	Neutral and acid mucopolysaccharides; glycogen	Oocyte coat Possible glycogen

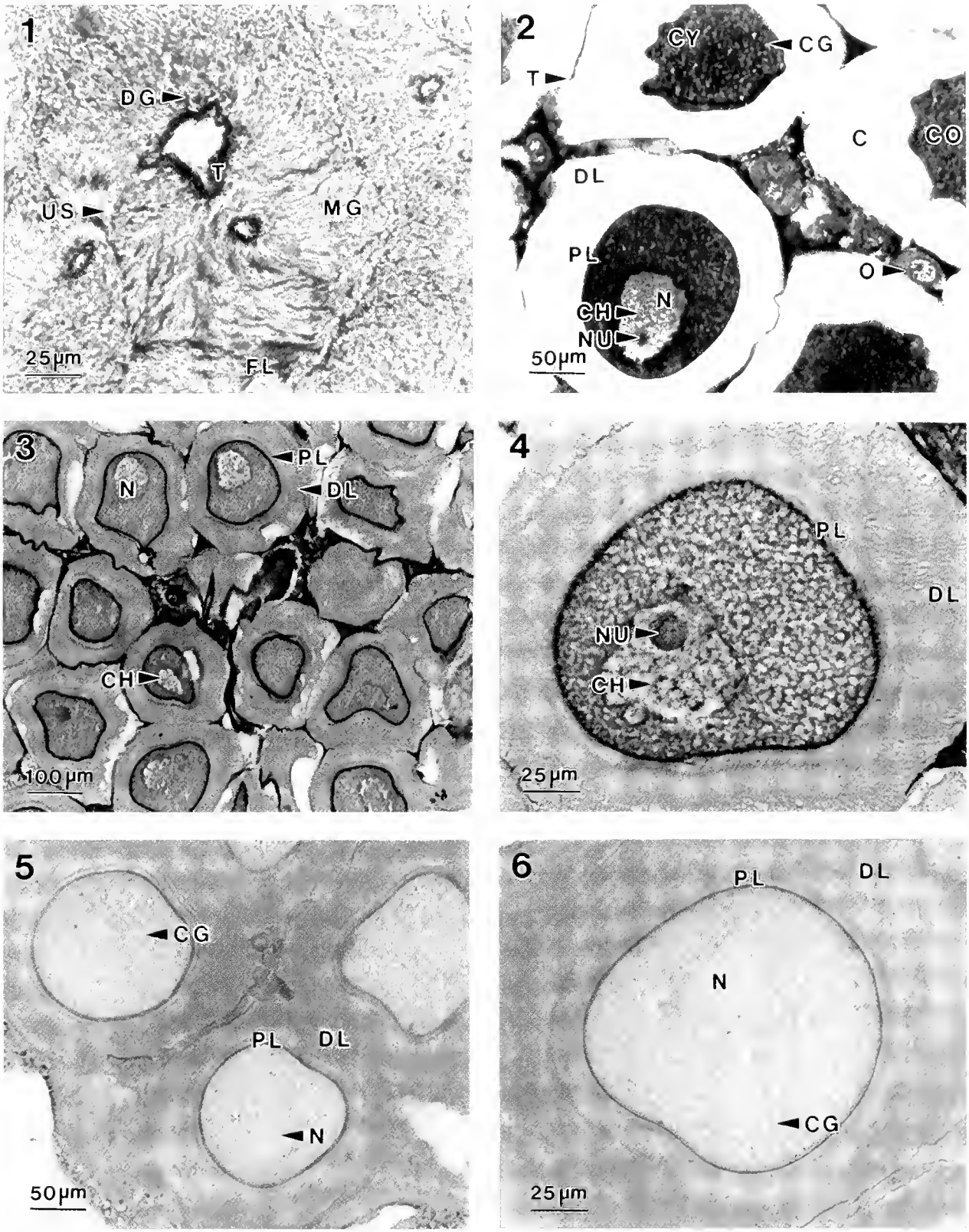

Figure 1. *M. crenulata* gonad. Photomicrographs of paraffin-embedded sections. Area (1) Histological section of male gonad. Modified Masson's trichrome protocol. T: trabecular tissue; DG: developing gametes; MG: mature gametes; FL: flagella; US: unoccupied space. Area (2) Histological section of female gonad. Modified Masson's trichrome protocol. N: nucleus; T: trabecular tissue; CO: coated oocyte; O: non-coated oocyte; C: coat; NU: nucleolus; PL: proximal layer of oocyte coat; DL: distal layer of oocyte coat; CY: cytoplasm; CH: heterochromatin; CG: unstained cytoplasmic globule. Area (3, 4) Female gonad. Trioxyhematein and alcian blue stains. DL: distal layer of oocyte coat; PL: proximal layer of oocyte coat; N: nucleus; NU: nucleolus; CH: heterochromatin. Area (5, 6) Female gonad. PAS-alcian blue protocol. N: unstained nucleus; DL: distal layer of oocyte coat; PL: proximal layer of oocyte coat; CG: cytoplasmic globule.

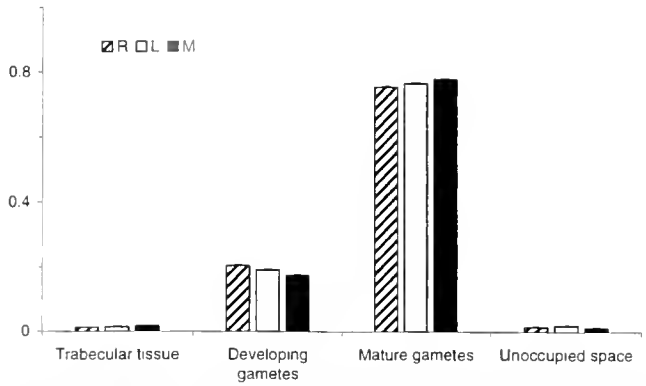

Figure 2. ***M. crenulata.*** **Volume fractions of tissue categories in right (R), left (L), and median (M) gonad regions of three males sampled in May 2000. The 95% confidence intervals are too small to be seen.**

ular tissue occupied a low and stable proportion of the testicle (approximately 0.03). Unoccupied space was rare (Fig. 3).

Females

Gonad Structure and Oocyte Histochemistry

The structure of the female gonad is shown in Figure 1, area 2. Trabeculae consisted of connective tissue (Fast Green positive) and were often masked by the acid fuschin. Uncoated oocytes adhered to the trabecular tissue and were small (approximately 25 μm) their cytoplasm was less intensely stained compared to coated oocytes. Unstained regions of trichrome-stained sections corresponded to the oocyte coat, which like the oocytes themselves appeared to be of constant dimension when the sectional plane passed through the nucleus. The cytoplasm of coated oocytes stained intensely with both trioxyhematein and acid fuschin, indicating the presence of numerous cytoplasmic globules. Many unstained globules (poorly visible in photographs due to the great staining heterogeneity of the sections) were present in the cytoplasm of the coated oocytes (Fig. 1 areas 2, 5, 6). Of the major biochemical tissue constituents, Masson's trichrome does not stain lipids (which are extracted during section preparation) or AMPS; as the globules were not alcian-blue positive (Fig. 1 areas 3–6), they were very probably lipid in nature.

The intensity of the cytoplasmic staining obscured the nucleus of those oocytes for which the sectional plane did not pass through the nucleus. When visible, the large nucleus (approximately 75 μm) presented dispersed heterochromatin and a single nucleolus (Fig. 1 area 2).

The oocyte coat appeared to be composed of two layers: a high-density proximal layer and a lower-density distal layer (Fig. 1 area 2). Staining with alcian blue confirmed this structure, and identified the principal coat constituent as AMPS (Fig. 1 areas 3–6). Counterstaining with trioxyhematein was extensive in the cytoplasm, indicating the presence of large quantities of nucleic acids, suggesting considerable anabolic activity (Fig. 1 areas 3, 4). The negative PAS reaction indicated both an absence of appreciable quantities of glycogen in the oocytes, and an absence of NMPS in the oocyte coat (Fig. 1 areas 5, 6).

Stereology

Ovarian homogeneity was verified in June, August, September, November and December 1999, and in February, April, May and June 2000. The near-identical volume fractions for the three identified ovary regions demonstrated the histological homogeneity of this organ (Fig. 4). These data confirm that the female gonad was structurally homogenous and gametogenetically synchronous. As was the case for the male gonad, representative histological data may thus be obtained from a single histological sample per individual. Similarly, the female gonad showed a stable tissue profile throughout the sampling period. Coated oocytes represented the great majority of the mean volume fraction (Fig. 5), from 0.83 (June 1999) to 0.94 (November 1999). Uncoated oocytes represented a small mean volume fraction, from 0.01 (November 1999) to 0.04 (June 1999). The mean volume fraction of trabecular tissue was also small: 0.035 (May 2000) to 0.12 (June 1999).

Oocyte Diameters

Given the homogeneity of the ovary, oocyte diameters were pooled for all females of a given sampling date. Mean diameters varied only slightly, from 125 to 135 μm without the coat (Fig. 6).

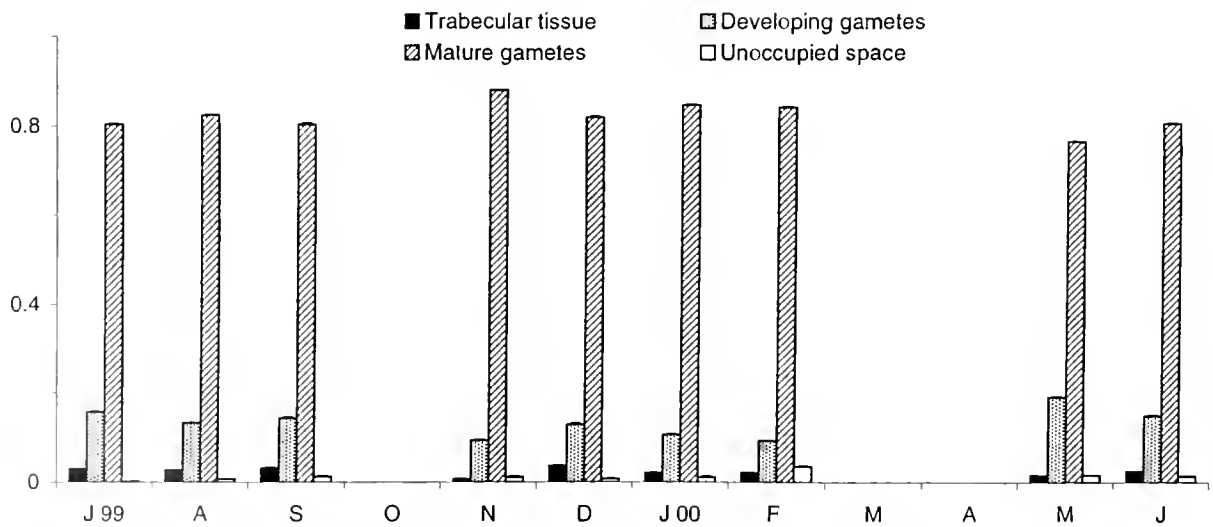

Figure 3. ***M. crenulata.*** **Evolution of tissue volume fractions in males, July 1999 to June 2000. The 95% confidence intervals are too small to be seen. Data unavailable in October, March, and April due to sampling difficulties.**

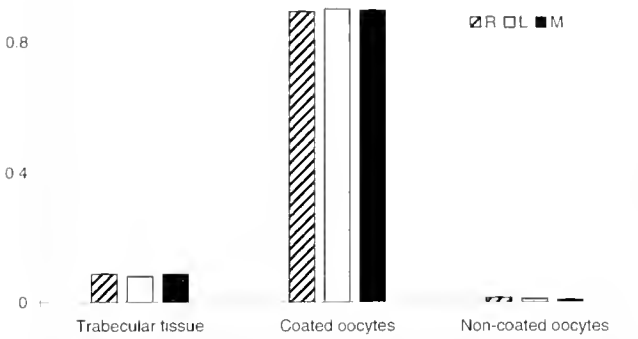

Figure 4. ***M. crenulata.*** **Volume fractions of tissue categories in right (R), left (L), and median (M) gonad regions of three females sampled in February 2000. The 95% confidence intervals are too small to be seen.**

This difference was not statistically significant (parametric ANOVA, normality and heteroscedasticity verified, $P \leq 0.05$).

DISCUSSION

To our knowledge, the results of the present study constitute the first report on the gonad structure, reproductive cycle and oocyte histochemistry in *Megathura crenulata*. A much more abundant literature exists for the commercially exploited archaeogastropods of the family Haliotidae, to which frequent reference will be made.

Gonad Structure

The gonad structure of *M. crenulata*, with gametes developing centrifugally from traversing trabeculae, resembles the well-known example of the Haliotidae (Newman 1967, Young & DeMartini 1970, Cochard 1980). In all of these cases, no ciliated evacuating ducts were observed; gametes are presumably expulsed via contractions of the gonad tegument, as is in *H. midae* (Newman 1967).

No simultaneous hermaphrodites were observed in any of the specimens studied; the dominant possible sexual modes for *M. crenulata* are therefore either gonochoric or successive hermaphrodite. Most prosobranchs are gonochoric, but there are a small number of hermaphroditic species (Fretter & Graham 1964, Fretter 1984). Complete resolution of this question in *M. crenulata* will require extensive sampling and long-term rearing.

Gonad structure was shown to be homogeneous for both male and female *M. crenulata*, as is also the case for *Haliotis midae* (Newman 1967). This result will facilitate future studies on the gonad of this species. By standardizing the histological sampling zone, it should be possible to reduce even further the residual inter-individual variation.

Reproductive Cycle

The marked stability of both the male and female *M. crenulata* gonad histological profile throughout the sampling period, as well as the uniform oocyte size, preclude the use of either criterion in determining gamete maturity, or even the state of spawning readiness of the gonad. The stable histological profile raises an interesting possibility: spawning readiness may depend on fine-tuning oocyte reserves rather than on synchronizing protracted periods of vitellogenesis. This is in contrast to the situation in the Haliotidae. A bimodal oocyte size distribution was observed in *H. midae* (Newman 1967); in *H. roei*, three oocyte maturation stages, characterized by different diameters, were observed (Shepherd & Laws 1974). Several oocyte sizes co-exist in *H. tuberculata*, with progressive growth from January to mid-July (Cochard 1980). Similarly, marked changes in histological profile characterize the reproductive cycle of *H. midae* males (Newman 1967). Whereas mature spermatozoa were found in *H. rufescens*, this appeared to correspond to a lack of variation in gonad indices and therefore year-round dribble spawning (Young & DeMartini 1970). Similarly, *H. asinina* synchronously spawns every two weeks on the Southern Great Barrier Reef (Jebreen et al. 2000). In the present case, the gonad appears ready to spawn at any moment in the reproductive cycle. The true state of gamete maturity must therefore be ascertained by other means, as suggested below.

A crude indicator of spawning readiness could involve determination of a gonosomatic index (DeVlaming et al. 1982); indeed, despite the uniform histological profile, considerable variations in gonad volume were observed over the sampling period in the few individuals dissected for histological processing. However, such a

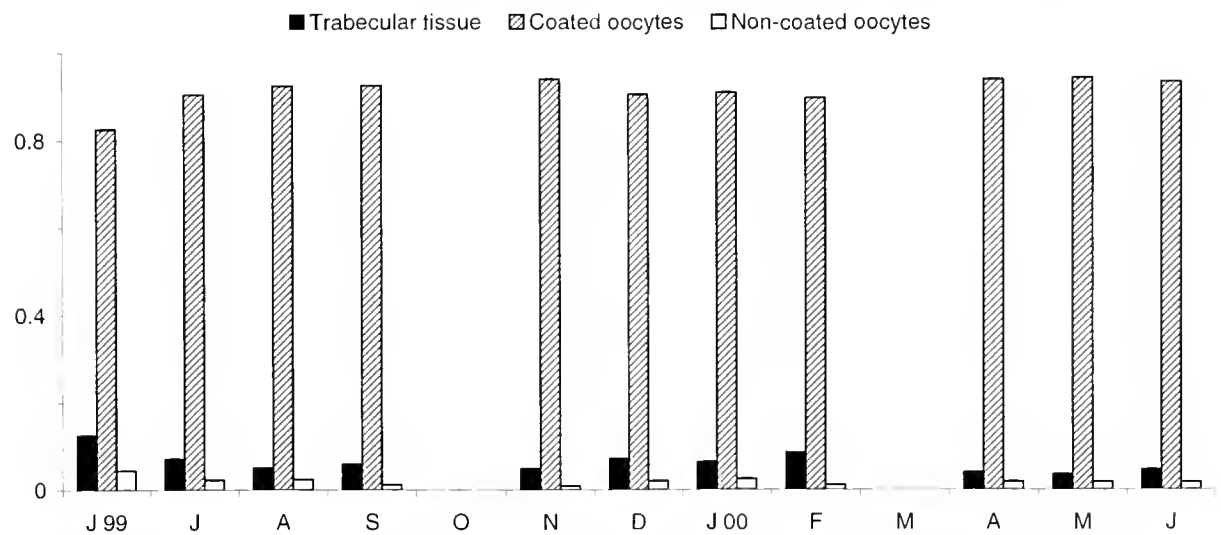

Figure 5. ***M. crenulata.*** **Evolution of tissue volume fractions in females, June 1999 to June 2000. The 95% confidence intervals are too small to be seen. Data unavailable in October and March due to sampling difficulties.**

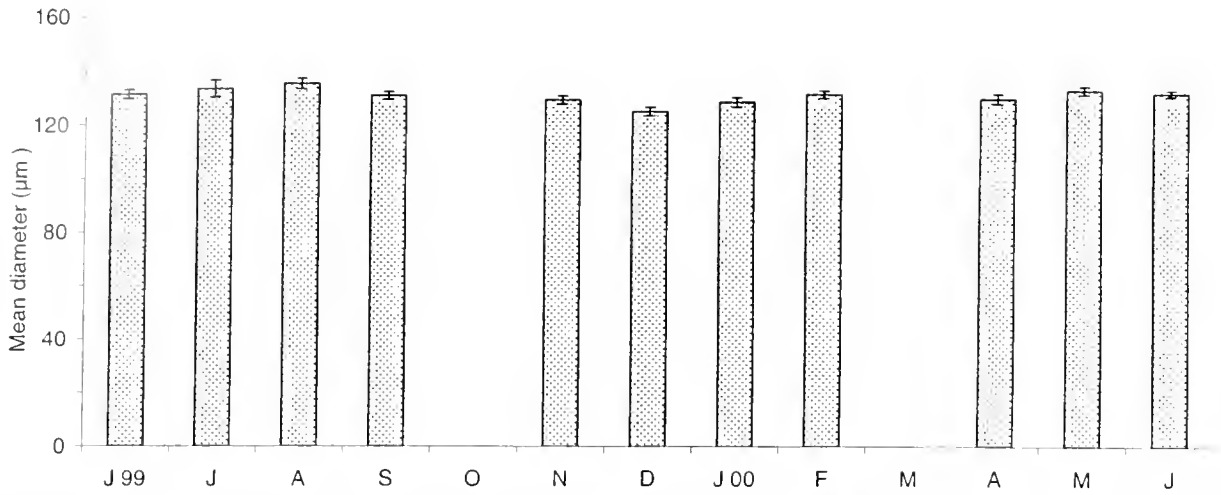

Figure 6. Evolution of *M. crenulata* coated-oocyte mean diameters (measured without their coats) from June 1999 to June 2000. Vertical bars are 95% confidence intervals. Data unavailable in October and March due to sampling difficulties.

technique requires the sacrifice of relatively large numbers of animals and is therefore not feasible in the context of the commercial exploitation of this species reared in captivity for repeated extraction of valuable hemolymph.

Oocyte Histochemistry

The presence of an oocyte coat, observed in mature oocytes of *M. crenulata*, is typical of archaeogastropods (Newman 1967, Cochard 1980). In gastropods with coated oocytes, gamones intervene in the modification of the coat to permit fertilization (Fretter & Graham 1964, Webber 1977, Fretter 1984). The results of the present study establish AMPS as a dominant component of the oocyte coat in *M. crenulata*. AMPS possess several chemical and mechanical properties which may confer important advantages to the oocytes: (i) Due to the high viscosity of AMPS (Beninger & St-Jean 1997, Davies & Hawkins 1998), they provide mechanical protection to the oocytes; (ii) AMPS reduce frictional resistance, thus allowing better water movement (Hoyt 1975, Daniel 1981, Davies & Hawkins 1998) over the egg masses, and hence improved gas exchange and metabolic waste removal. The reduced frictional resistance would also reduce the probability of dislodging the egg masses; (iii) AMPS possess anti-microbial properties (Sasikala & Subramoniam 1987, Subramoniam 1991, Beninger & Larocque 1998), potentially conferring protection from opportunistic microbes in the egg masses; (iv) the relatively high density of the AMPS coats could act to confer negative buoyancy to the otherwise positively buoyant (due to the high lipid content) oocytes, allowing the egg masses to remain on the substrate rather than in the water column. This characteristic is important in species which limit propagule dispersal; further studies on the reproductive biology of *M. crenulata* could address this possibility; (v) AMPS adhere to and agglutinate particles strongly, as shown in the context of bivalve particle processing (Beninger & St-Jean 1997). This property would once again reduce the dispersal of the oocytes, which are oviposited as egg masses.

The lack of positive PAS staining in the oocyte cytoplasm eliminates the possibility of glycogen as a reserve in the oocytes of this species. The chief oocyte reserve appears to be lipid (visible as clear globules in the trichrome-stained sections), as is the rule in the Mollusca (Gallager & Mann 1986, Lucas et al. 1986, Caers et al. 1999, Lu et al. 1999). Large lipid reserves have been reported in the ovaries of both *Haliotis* and *Megathura* genera (Webber 1977).

Although histological examination is a well-established technique for the detailed documentation of reproductive cycles (Webber 1977, Beninger 1987, Barber & Blake 1991), the results of the present study show that this approach, while very useful for elucidating other aspects of the reproductive biology of *M. crenulata*, cannot be used to follow and pinpoint spawning preparedness in this species. However, the eventual rearing of *M. crenulata* will require this information; even more desirable would be a non-destructive technique of monitoring the reproductive status of broodstock. Such biological monitoring of broodstock could lead to increased fertilization success, and hence increased production of adults for pharmacological use.

Several additional aspects of the reproductive biology of *M. crenulata* which could be usefully pursued include the dynamics of gametogenesis (especially the transition from small uncoated oocytes to large coated ones), the buildup of vitteline reserves, the characteristics of gamete storage, and the mechanisms of gametogenetic synchronization. Such information will be most helpful in both the management of wild stocks, and in future aquaculture operations.

ACKNOWLEDGMENTS

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MODELING THE GROWTH OF THE CHILEAN LOCO, *CONCHOLEPAS CONCHOLEPAS* (BRUGUIÈRE, 1789) USING A MODIFIED GOMPERTZ-TYPE FUNCTION

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ABSTRACT A modified Gompertz-type function was fitted by nonlinear numerical methods to the absolute growth rates(mm/day) of the marine gastropod *Concholepas concholepas*, known in Chile as "loco." The data for this study were obtained from laboratory and field experiments as well as from published and unpublished reports of loco growth. The fit of the modified Gompertz-type function was compared to the fit obtained with the commonly used von Bertalanffy function using the Akaike information criteria corrected by sample size bias (AIC_c). The modified Gompertz function used in this study was $dL/dt = (a_0 L^{a1})*(exp^{-a2t}) + a_3$, where L is the peristomal length (mm), and a_0, a_1, a_2 and a_3 are the parameters of the model. The biological meaning of the constant a_3 of the function is equivalent to the asymptotic growth rate for the species. The von Bertalanffy growth rate equation can be expressed as $dL/dt = -k(L_\infty - L)$. This equation represents a straight line with a negative slope equal to $-k$ and with the y-axis intercept $dL/dt = 0$, providing an estimate of L_∞. The fitted parameters obtained by fitting a modified Gompertz-type of model to the pooled growth rate data were $a_0 = 2.9300e{-}05$, $a_1 = 4.2605$, $a_2 = 0.2011$, and $a_3 = 0.02852$ with a degree of freedom corrected $r^2 = 0.595$. For the von Bertalanffy function the fitted parameters were $k = 5.943e{-}04$ (day^{-1}) and $L_\infty = 150.6$ mm with $r^2 = 0.387$. The $AICc$ of the Gompertz fit was −6,086; whereas, the $AICc$ of the von Bertalanffy fit was −5,611. The smaller AIC_c value −6,086 indicates that the modified Gompertz function provides a better fit to the loco growth rate data. The form of the projected modified Gompertz-type growth curve differs notably from the traditional von Bertalanffy curve for juvenile growth. The modified Gompertz model does not reach an asymptotic growth level, predicting instead infinite growth. This latter property could easily explain reported historical findings of loco individuals measuring over 170 mm in peristomal length.

KEY WORDS: Growth rate, Gompertz model, intertidal gastropods, aquaculture

INTRODUCTION

The loco (*Concholepas concholepas* Bruguière, 1789) is a muricid gastropod endemic to the Southeastern Pacific, found along the coasts of Perú and Chile (Bustos *et al.* 1986). Because of the great economic importance attained by the loco in the past two decades, its natural stocks have been under intense exploitation pressure. This has prompted the Chilean Government to enact legal measures aimed at protecting this species by regulating the capture effort and by establishing a minimum legal size of extraction (100 mm, peristomal length) (Montt *et al.* 1977). The locos are mainly exploited by the artisanal sector, which adds a social dimension to its economic importance. The factors above have made the loco one of the better-studied marine gastropods in Chile, with most of the research effort directed toward commercial farming of this species.

Growth-rate studies of loco juveniles have been carried out in the laboratory (Méndez & Cancino 1992), in suspended lantern nets (Varela & Pérez 1987), and in the field, especially in intertidal rocky shores (Guisado & Castilla 1983, Bustos *et al.* 1985, Bustos et al. 1986, Adlerstein 1986). The early studies of age and growth of loco were initially descriptive and were not used to produce mathematical growth models (Tobella 1975, Lozada *et al.* 1976, Acuña & Stuardo 1979). Later studies were oriented toward satisfying the management requirements of the loco fishery. Initially, several authors applied linear functions to describe the growth of loco juveniles (Guisado & Castilla 1983). More recently, Reyes and Moreno (1990) employed linear and exponential models to describe the growth of juveniles under 10 mm length from the Mehuín marine reserve in the X Region of Chile (Fig. 1). However, the function most widely used to model loco growth and to regulate its fishery is the von Bertalanffy (1957) growth equation (Bustos *et al.*1985, Bustos *et al.* 1986, Adlerstein 1986, Castilla & Jerez 1986, López 1987).

The von Bertalanffy growth equation can be expressed as $dL/dt = -k(L_\infty - L)$. This differential equation represents a straight line with a negative slope equal to $-k$ and with the y-axis intercept $dL/dt = 0$, providing an estimate of L_∞. Use of the von Bertalanffy equation is limited, because often it does not adequately reflect the growth of immature or juvenile individuals. In addition, in its original form, the von Bertalanffy equation does not include the seasonal oscillation of growth, an important factor if the growth of organisms is to be described on short time scales (Pauly & Gaschütz 1979, Gaschütz *et al.* 1980, Akamine 1986, Allison 1994, Askew 1995) and specifically for intertidal gastropods, (Ekaratne & Crisp 1984, Santarelli & Gros 1985, Noda 1991, Shepherd *et al.* 1995).

An alternative to the von Bertalanffy function is the Gompertz function, which in a modified version, has been used to model individual growth rates of juvenile and adult abalones (*Haliotis asinina*) (McNamara & Johnson 1995), pre- and postlarval stages and adult fishes (Smith & Kostlan 1991; Williams & Lowe 1997; Zweiffel & Lasker 1976) and crustaceans (Misra 1957).

In this paper, data from growth experiments of loco individuals grown in the laboratory and in suspended lanterns, as well as data obtained from a wide variety of literature sources, were pooled together employing meta-analysis techniques. The use of meta-analysis makes it possible to integrate and summarize information coming from a wide variety of sources. As applied to stock assessment, meta-analysis involves the compilation of pre-existing

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Figure 1. Geographic distribution of *Concholepas concholepas* in Chile and location of study area Quintay Bay, V región.

datasets to determine the values of parameters of models or to develop prior probability distributions of these parameters (Cooper & Hedges 1994, CFSAM 1998). This approach permits a test of the hypothesis that a modified Gompertz equation provides a better model of the loco growth curve than the commonly used von Bertalanffy function. It is hoped that the development of more accurate loco growth models will improve the management of this commercially and socially important marine resource.

MATERIALS AND METHODS

The data used in this study were taken from growth studies of loco juveniles carried out in laboratory conditions and in suspended lanterns anchored in the Quintay Bay (33°11′ S; 71°42′ W), V Region of Chile (Fig. 1). Loco juveniles were collected from the intertidal rocky shore. Individuals were marked with circular plastic tags (2-mm diameter) and the peristomal length (Fig. 2) was measured with a precision caliper (± 0.05-mm).

The laboratory growth experiments of loco juveniles were carried out in two 40-L glass tanks (0.5 × 0.4 × 0.4), using the facilities of the School of Fisheries, Universidad del Mar. Each tank was furnished with independent airflow systems powered by Elite 800 electrical pumps with a 2,100-cc air/min capacity. During the study, the water temperature in the aquarium tanks was 20° C ± 2°C. Growth controls throughout the study were taken on days 19, 34, 55, 69, and 90. Loco individuals grown in laboratory and in culture systems were fed *ad libitum* with a monospecific diet, based on live intertidal mussels, *Perumytilus purpuratus* (Lamarck 1819), one of the main prey items of the locos in their natural environment (Castilla et al. 1979). The initial peristomal length-size range of the locos grown in laboratory conditions was 24 to 64 mm.

The growth experiments in suspended lanterns were carried out in the Quintay Bay during two periods lasting 54 and 140 days, respectively. The loco growth lanterns were especially designed, having a hard plate made of fiberglass that offered an anchor substrate to the locos (Fig. 3). The initial peristomal size range of the loco juveniles grown in lanterns was in the same peristomal size range (24 to 64 mm).

The size increments were calculated using the absolute growth rate, mathematically represented by:

$$\frac{\Delta L}{\Delta t} = \frac{L_f - L_i}{t_f - t_i} \tag{1}$$

where L_f and L_i are the final and initial peristomal lengths and t_f and t_i are the final and initial times corresponding to the aforementioned lengths. The calculated growth rates values were fitted

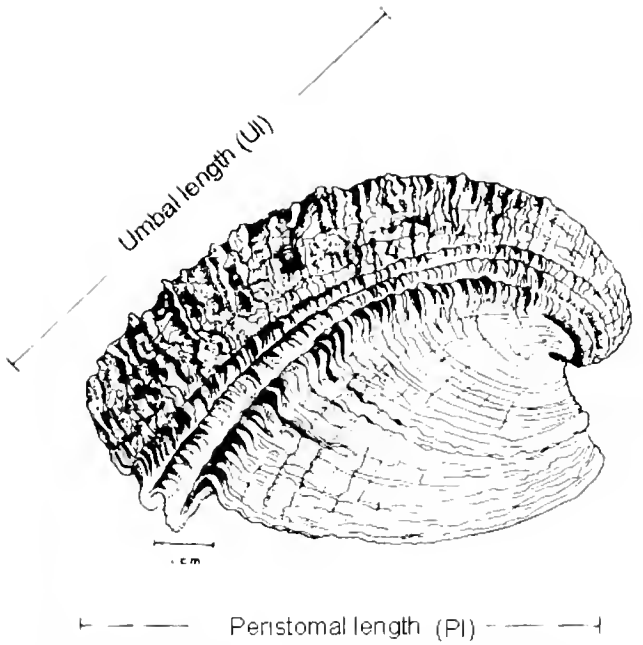

Figure 2. Diagram showing a loco (*Concholepas concholepas*).

to the modified Gompertz and von Bertalanffy functions using the Levemberg – Marquardt algorithm from the Table Curve 4.0 package (SPSS 1998).

The modified Gompertz function is expressed as:

$$\frac{dL}{dt} = (a_0 L^{a1}) * (\exp^{-a_2 L}) + a_3 \qquad (2)$$

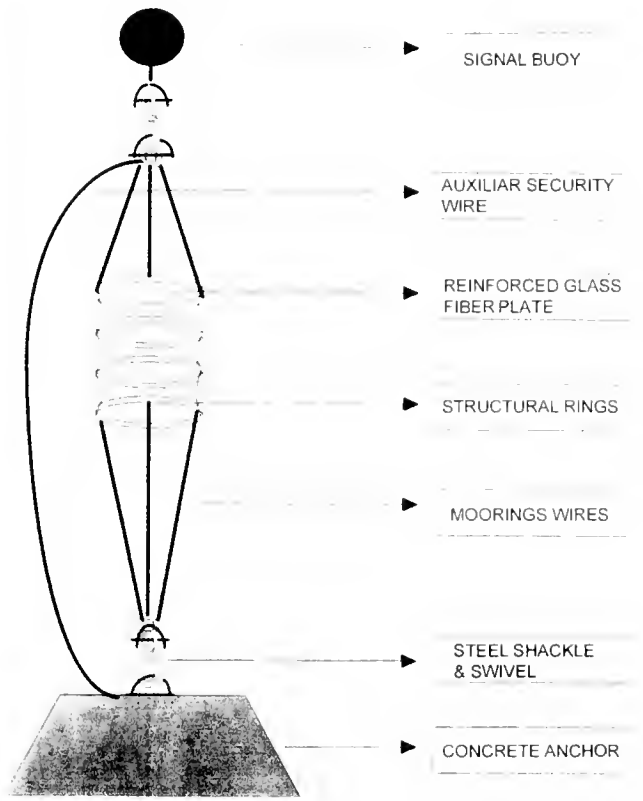

Figure 3. Diagram of the suspended lantern net designed to grow locos in natural conditions.

where L is peristomal length (mm) and, a_0, a_1, a_2, and a_3 are the fitting parameters where a_3 is equivalent to the asymptotic growth rate for the species. The first term of this expression ($a_0 L^{a1}$) is analogous to the power allometric equation proposed by Parker and Larkin (1959); whereas, the second ($exp^{-a2 L}$) is a factor that produces a decreasing growth rate beyond the maximum value of the function. This inflection point is represented by the size of individuals at which sexual maturity is reached.

The von Bertalanffy function is expressed as:

$$\frac{dL}{dt} = -k^*(L_\infty - L) \qquad (3)$$

where L_∞ and k are the asymptotic peristomal length and the curvature parameters, respectively.

The selection of the best-fit growth curve was made based on information theory (Akaike1974, Burham & Anderson 1998). The specific tool used for model comparison and selection was Akaike's information criteria corrected for sample size bias, AIC_C defined as:

$$AIC_C = n^*\log_e(MSE) + 2K + \frac{2K(K+1)}{(n-K-1)} \qquad (4)$$

where *MSE* is the mean square error from the model, n is the number of datapoints, and K is the number of parameters to be estimated. The first term of this expression reflects the lack of fit, the second is a penalty term for complexity or lack of parsimony of the model, and the third term is a correction factor for sample size bias. Lower AIC_C values indicate a better model fit (Burham and Anderson 1998).

If we know the functional relationship between growth rate and length, an individual's body size as a function of age or time frame can be obtained by reorganizing Eq. 2 and integrating:

$$\int_0^t dt = \int_{L_0}^{L_t} \frac{dL}{(a_0 L^{a1}) * (\exp^{-a_1 L}) + a_3} \qquad (5)$$

where L_t is the final peristomal length (mm) of the individual at age or time t, and L_0 is the initial peristomal length (mm) of the individual at the beginning of the experiment or when $t = 0$. Equation 5 cannot be solved analytically; however, a solution can be obtained numerically using a numerical integration subroutine available in any specialized mathematical software package.

Because it may be convenient to use a simpler and more practical method for growth projections in the farming business, we also use a recursive-type approximation based on a discrete time interval Δt, which is mathematically represented as:

$$L_j \approx L_{j-1} + \sum_{i=1}^{30} (a_0 L_i^{a1} * \exp^{-a_2 L_i} + a_3) * \Delta t \qquad (6)$$

where L_j is the peristomal length in the j-th period, and L_{j-1} is the peristomal length in the $(j-1)$-th period. The term between parentheses is the growth rate expressed in mm/day of an individual in the $(j-1)$-th period, which is dependent on length and the discreet increment $L_i = (L_{i-1} + \Delta L_{i-1})$, and Δt is the timestep interval in days. The timestep utilized was one day, and the unit growth period utilized was monthly.

The loco growth data from this study were pooled with published and unpublished loco growth datasets (Table 1). This al-

TABLE 1.

Published and unpublished data sources for growth rate of locos used in this study.

Data source (author and year)	Peristomal size range (mm)	Growth rate range or average (mm/day)	Locality (region)
Rabi & Maravi (1997)	25–88	0.115 SD = 0.079	Ilo Port
		0.107 SD = 0.050	Catarindo Bay (Perú)
Stotz & Pérez (1992)	10–90	0.016–0.250	Tagged *in situ* Pta. Lagunillas (IV)
Gallardo (1979)	1.8–30	0.104	Modal progression and tagged Mehuín (X)
Gnocochea et al. (1991)	<10	0.130	Aquaria laboratory
	>10	0.103	
Castilla (1983)	14–15	0.100	Mehuín (X)
	14–15	0.110	Caleta Leandro (VIII)
López (1987)	Not available	0.041–0.071	Tagged *in situ* Mehuín (X)
López et al. (1991)	7.05	0.061	Ramuntcho (VIII)
Olivares et al. (1990)	8–20	0.018–0.054	Aquaria laboratory (VIII)
Varela & López (1989)	Not available	—	Suspended system Aquaria laboratory
Lozada et al. (1976)			Modal progression
	54–63	0.073–0.200	Pta. Saliente (IV)
	63–81	0.100–0.106	Caleta Leandro (VIII)
	63–90	0.100	Talcahuano (VIII)
Rivas (1994)	44–64	0.039–0.071	Suspended system Caleta Lirquén (VIII)
Mora (1994)	36–65	0.063–0.073	Suspended system Caleta Lirquén (VIII)
Tobella (1975)	14–113	0.045–0.300	Modal progression Caleta Leandro (VIII)
Reyes & Moreno (1990)	1.9–10	0.022–0.058	Modal progression Mehuín (X)
IFOP (1992)	10–145	0.008–0.086	Tagged *in situ* Punta Corona (X)
		mean = 0.0284	
		SD = 0.0197	
Acuña & Stuardo (1979)	20	0.100	Modal progression Montemar (V)
Guisado & Castilla (1983)	11–49	0.05–0.232	Modal progression Las Cruces (V)
Castilla & Jerez (1986)	40–110	0.013–0.050	Las Cruces, El Quisco, Quintay (V)
Mendez & Cancino (1992)	5–12	0.046–0.075	Aquaria laboratory Las Cruces & El Quisco (V)
This study	24–64	0.015–0.117	Suspended system Quintay Bay (V)
		mean = 0.070	
		SD = 0.027	
	24–64	0.020–0.093	Aquaria laboratory
		mean = 0.038	
		SD = 0.014	

lowed a substantial increase in the size distribution of the data fitted to the von Bertalanffy and the modified Gompertz growth function.

RESULTS AND DISCUSSION

The average growth rate of loco individuals in the laboratory (0.038 mm/day and standard deviation 0.014 mm/day), was about one-half the average growth rate for individuals grown in suspended lantern nets in the field (0.070 mm/day and standard deviation of 0.027 mm/day). The growth rate ranges observed in the laboratory and in the field are, however, in agreement with growth rates reported for other muricid species by Spight *et al.* (1974). The observed differences between growth rates in the laboratory and suspended lanterns probably reflect better growth rate conditions in the field. The locos grown in suspended lanterns were subjected to lower and more variable changes of ambient temperature (range 10 C –20°C) than the locos grown in the laboratory. Higher temperatures have been associated with higher growth rates in locos grown under laboratory conditions (Méndez & Cancino,1992); therefore, the higher growth rates obtained under colder field conditions would indicate that factors other than temperature should be used to explain the observed growth rate difference between laboratory- and lantern-grown locos. This conclusion must be reached with caution, however, because the results reported by Méndez & Cancino (1992) (Table 1) are for juvenile locos below the 20-mm peristomal size length; whereas, the present study included individuals in the 24 to 64-mm size range. For the same reason, growth rates obtained in the laboratory in this study are not directly comparable to other laboratory studies of

TABLE 2.

Fitted modified Gompertz and von Bertalanffy parameters for loco growth rates.

Function	Parameters	Standard error	*t*-value	AIC_C
Gompertz modified	a_0 = 2.69e-05	1.625e-05	1.654717	−6086.8
	a_1 = 4.094	0.276217	14.82061	
	a_2 = 0.183	0.010620	17.25293	
	a_3 = 0.0281	0.000777	36.00687	
Von Bertalanffy	k = 5.943e-04	2.722d-05	21.82896	−5611.1
	L_∞ = 150.60	3.138003	48.00212	

Note. The *t*-values serve as indicators of the degree of certainty with the parameters are determined. The parameter with the highest *t*-value, in addition to having the greatest contribution to the fit, will also been determined with the greatest level of certainty (SPSS 1998).

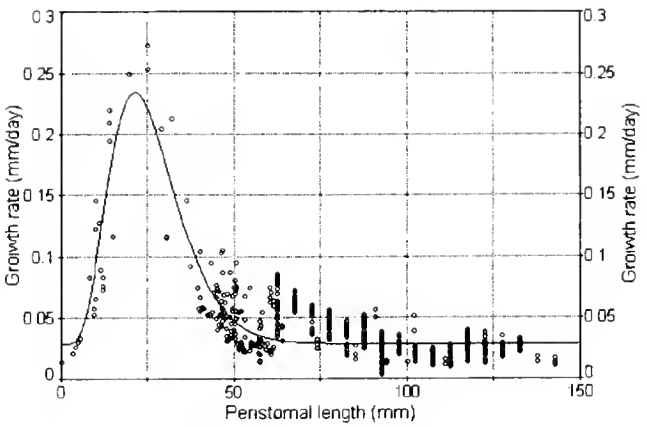

Figure 4. Gompertz curve fit to growth rate of locos.

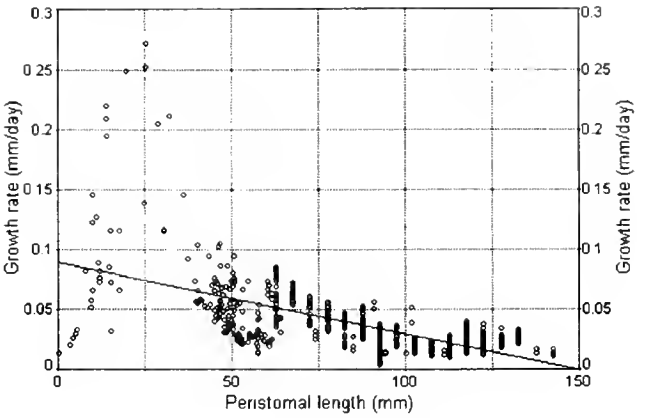

Figure 5. Von Bertalanffy curve fit to growth rate of locos.

loco growth reported by other authors: 0.103 – 0.130 mm/day (Goicochea *et al.* 1991), 0.018–0.058 mm/day (Olivares *et al.* 1990) and 0.046 – 0.075 mm/day (Méndez & Cancino 1992), mainly because these authors worked with loco individuals below the 20-mm size range. Reyes and Moreno (1990) obtained values from 0.022 to 0.043 mm/day for natural loco populations (<20 mm umbal lengths) from the Mehuín marine reserve in the X Region of Chile. This would indicate that environmental pressure can depress the potential juvenile loco growth in the field.

The growth rates reported here are in better agreement with results reported by Rabí and Maraví (1997) for culture experiments in Peru and with growth data reported by Castilla and Jerez (1986) for natural loco population in the V region of Chile. Similar growth rates (0.039–0.073 mm/day) were obtained by Mora (1994) and Rivas (1994) in culture experiments in Caleta Lirquén in the VIII region of Chile (Table 1).

The fitted parameters for the pooled growth rate data obtained during this study and from published and unpublished data for the Gompertz function (Table 2 ; Fig. 4) were a_0 = 2.9300e-05, a_1 = 4.2605, a_2 = 0.2011, and a_3 = 0.02852 with a degree of freedom corrected r^2 = 0.595 and AIC_C = –6,086. For the von Bertalanffy function (Table 2 ; Fig. 5), the fitted parameters were k = 5.943e-04 (day $^{-1}$) and L_∞ = 150.6 with r^2 = 0.387 and AIC_C = –5611. The lower AIC_C value for the modified Gompertz function indicates that this function provides a better fit. Although the corrected r^2 only accounts for 59.5% of the variability of the data, this is, nonetheless, better than the 38.7% obtained with the von Bertalanffy model.

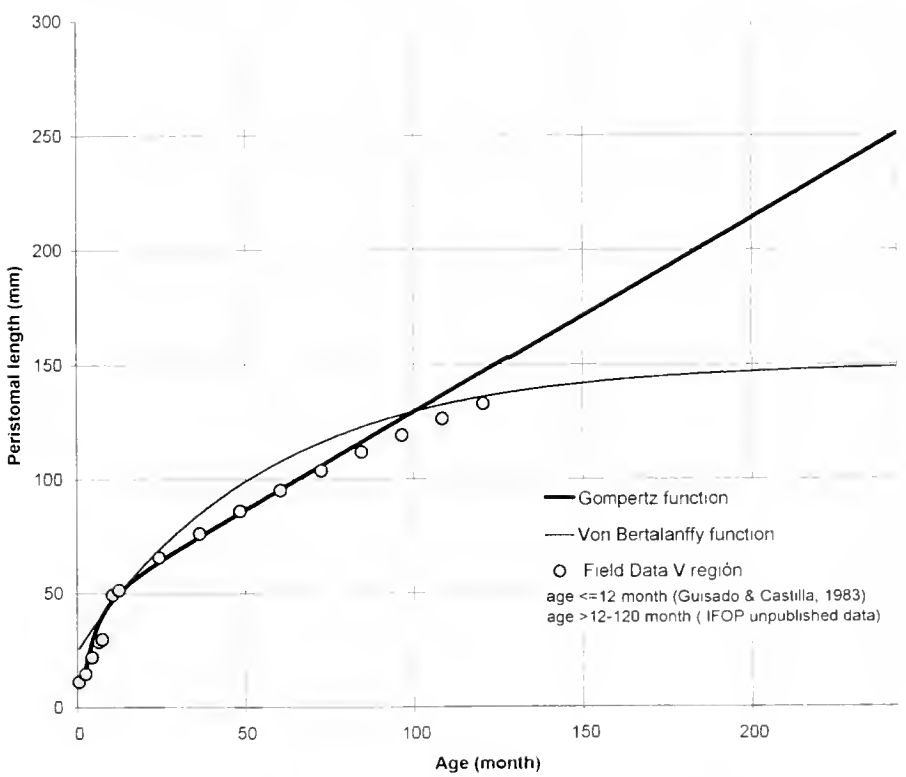

Figure 6. Growth curve of peristomal length (mm) at age (months), for locos measured in natural enviroment. Gompertz function (- - -) and von Bertalanffy function (-.-.-).

The inflection point of the fitted growth-rate curve or point of maximum growth rate (Fig. 4) represents the individual's change of state from a sexually immature to a sexually mature individual. The energy budget of immature individuals is exclusively destined to growth, but when sexual maturation occurs, individuals redirect energy toward the production of gonads, resulting in a marked reduction in growth rates. This inflection point indicates that locos reach sexual maturity at about 25 to 30-mm peristomal length. This is well below the 50-mm minimum size value reported by Ramorino (1979), and it may well reflect a behavioral shift toward earlier time of sexual maturity in the loco population, which, during the last two decades, has been subject to a more intense level of exploitation.

A growth projection of this species was performed as a function of time by using the discrete character expression represented by Eq. 6 and compared to the same projection of the fitted von Bertalanffy growth curve from this study (Fig. 6). For the sake of comparison, the sizes of individuals under a year reported by Guisado and Castilla (1983), and the mean length between one and ten years (IFOP unpublished data) for the Region V are superimposed.

The growth projection using the modified Gompertz function indicates that the legal size of capture of 100-mm peristomal length, should be reached in 66 months. The modified Gompertz-type model does not reach an asymptotic size as predicted by the von Bertalanffy expression. Instead, the projected growth, especially for individuals over a year old, has a resulting curve that is notably similar to the infinite growth pattern described for some other marine invertebrates (Tanaka 1982, Tanaka 1988, Phillips *et al.* 1983, 1992, Ariyama 1993, McNamara & Johnson 1995). This could easily explain historical findings of loco individuals measuring over 170 mm in peristomal length. Furthermore, the fitted von Bertalanffy curve does not adequately describe the juvenile loco growth pattern. Our results indicate that the modified Gompertz equation provides a stronger model of the loco growth curve than the commonly used von Bertalanffy function. Based on these results, we propose that the modified Gompertz equation described in this study should be used routinely in the management models of the commercially and socially important loco species.

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UNCOVERING ECONOMIC BENEFITS OF CHIVITA (*MELONGENA MELONGENA* LINNAEUS, 1758 AND *MELONGENA CORONA BISPINOSA* PHILIPPI, 1844)

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ABSTRACT West Indian crown conch *Melongena melongena* Linnaeus and other crown conch e.g., *Melongena corona* Gmelin have been viewed by most researchers and marine resource beneficiaries as predatory species with little or no value. Focus groups and individual interviews were conducted with local residents as part of the design phase for an economic valuation study of mangrove ecosystems in Yucatán, Mexico. They examined how local inhabitants use, perceive of, and understand ecological services associated with their shared mangrove ecosystem. The data revealed that collection of *Melongena melongena* and *Melongena corona bispinosa* Philippi (collectively called "chivita") has become an increasingly significant component of these communities' economic activity. It was learned that chivita collection is replacing other marine resource-based subsistence strategies in these communities. These findings place *Melongena melongena* and *Melongena corona bispinosa* in a new light.

KEY WORDS: Mangroves, qualitative methods, economic value, Mexico

INTRODUCTION

The West Indian crown conch (*Melongena melongena* Linnaeus, 1758) is generally thought of as a predatory species that preys on other "valuable" species such as the oyster (*Crassostrea virginica*, Gmelin) (Villarreal 1989). The published research on the West Indian crown conch and its close relative the Florida crown conch (*Melongena corona* Gmelin) has centered on the predatory nature and behavior of these species (Bowling 1994, Dalby 1989, Ellison and Farnsworth 1992, Garcia-Cubas 1981, Villarreal 1989). Some of the only reported research on *Melongena corona bispinosa*, Philippi, 1844 appears as part of conference proceedings several years ago and centers on the reproductive life of the conch (Manzano et al. 1998; Zárate and Arana 1998; Zárate et al. 1998). In Mexico and in this paper, the West Indian crown conch (*Melongena melongena L.*), Caracol Negro [Black conch] (*Melongena corona bispinosa*), and the Florida crown conch (*Melongena corona*) are collectively referred to as "chivita" (Secretaria de Medio Ambiente Rucursos Naturales y Pesca [SEMARNAP] 1995). While there have been a few passing references to some economic value associated with chivita, there appear to be no documented studies in the literature on economic, ecological, or social benefits associated with these shellfish.

In several instances, researchers have made reference to some commercial value associated with chivita. Villarreal (1989) makes the passing remark that in Veracruz, Mexico, "fishermen . . . believe that [*Melongena melongena*] should be exterminated. On the other hand, since this snail is of economic importance, its capture is considered more attractive." However, Villarreal Chavez does not support, amplify, or explain what is meant by this. Elsewhere, Solfs-Ramfrez (1994, 20) includes chivitas (*M. melongena* and *M. corona*) as commercially exploitable shellfish species in one of that author's tables on the mollusks of the Yucatán Peninsula. Unfortunately, Solfs-Ramfrez does not refer to chivita in the article's text nor further explain the chivita reference. Most recently, Zetina Zárate et al. (1998), in their introductory remarks on the reproductive cycle of *M. corona bispinosa*, make otherwise unsupported statements to the effect that the collection of chivita is important and subject to increasing collection pressures. As a result of the absence of information on economic benefits associated with chivita collection, this paper provides some useful information on the economic significance of the collection of West Indian conch and Black crown conch (chivita) in coastal communities in northern Yucatán, Mexico.

Hypotheses

Complex environmental and natural resources, such as the Yucatán's mangrove wetlands and coastal resources, represent substantial sources of cultural, intergenerational, environmental, and economic wealth (Aylward and Barbier 1992, Bann 1997, Barbier 1994, Barbier et al. 1997, Carson 1998, Perrings 1995). As part of an effort to identify the range of relevant ecosystem services for a study of the economic value of mangrove ecosystems in northern Yucatán, Mexico, I undertook a series of qualitative inquiries. Two qualitative research methods were used—focus groups[1] and individual interviews.[2] This paper examines research questions concerning the economic significance of chivita collection in two coastal fishing communities bordering Chelém Lagoon near Progresso, Mexico. This paper focuses on how local beneficiaries perceive, use, and value chivita. First, the hypothesis is explored that chivita collection is a positive economic activity for the communities of Chelém and Chuburná. A second hypothesis examined whether there is a significant positive economic benefit associated with chivita collection. It should be noted that findings concerning conflicting management agendas and the relative merit of the use of focus group and individual interview data for resource valuation are presented elsewhere (Kaplowitz and Hoehn 2001, Kaplowitz 1999, Kaplowitz 2000a, Kaplowitz 2000b). This paper briefly describes the research design and locale before discussing the collection method and analysis of data on the economic significance of chivita collection. Finally, the results are

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[1]Focus groups are carefully planned discussions designed to learn about subjects' perceptions on a defined area of interest in a permissive, nonthreatening environment. They are conducted by a skilled moderator who follows a discussion guide and involve as few as 2 to as many as 12 informants.

[2]Individual interviews (also called unstructured, exploratory, intensive, in-depth, and depth interviews) are guided conversations whose goal is to elicit from interviewees (also called informants) rich, detailed materials that can be used in qualitative analysis. The interviewer used the same discussion guide as used in focus groups to guide the one-on-one conversations.

discussed before concluding with some observations and implications of the findings.

MATERIALS AND METHODS

Researchers in diverse fields of study regularly use qualitative methods such as, focus groups and individual interviews, as comprehensive research tools and as important components in designing and implementing reliable research studies (Chilton and Hutchinson 1999, Krueger 1994, Morgan 1997, Schwarz 1997, Sudman et al. 1996, Weiss 1994). Qualitative methods for analyzing and managing shellfisheries have been reported previously (Whitlatch and Osman 1994). Individual in-depth interviews are efficient means for collecting information on beneficiaries' use and understanding of mangrove ecosystems at the local level (Kovacs 1999). Furthermore, focus groups and individual interviews have proven to be complementary in identifying conflicting resource agendas (Kaplowitz 1999). They are also useful for learning from local beneficiaries how they use, perceive, and value environmental and natural resources (Kaplowitz and Hoehn 2001, Kaplowitz 2000a, Mandondo 1997). Studies have also shown that resource beneficiaries' ideas about and use of natural resources may differ greatly from those of scientists and so-called experts (Talawar and Rhoades 1998).

Locale and Procedure

The communities of Chelém and Chuburná, Mexico are located along a 15-kilometer stretch of coastal fringe that borders the Gulf of Mexico on one side and Chelém Lagoon on the other. These communities are, respectively, about 3 and 15 kilometers west of the port city of Progresso. The villages are comprised of families that have traditionally relied upon the natural resources of the region, including the mangrove wetland, for their subsistence and livelihood. The inhabitants of Chelém and Chuburná share similar socio-economic characteristics and have roughly 475 and 215 households respectively (Instituto Nacional de Estadfstica Geograffa e Informática [INEGI] 1992). Traditionally, these communities have relied upon a combination of activities for their subsistence and economic gain. They have been able to survive long periods (November to March) of seasonal bad weather "nortes" by developing a multiple use and activity strategy of combining fishing in the sea and lagoons, small scale salt extraction, agriculture, and tourism activities (Paré and Fraga 1994).

A total of 97 year-round residents from the two communities were interviewed in one of 12 focus groups or 19 individual in-depth interviews. The research design allowed for examination of the collected data across interview type, gender, and community. Research assistants canvassed randomly and selected sections of the target communities at staggered times of day to recruit participants. The focus groups were comprised of between four and seven individuals of the same gender from the same village. No respondent or their family members participated in more than one focus group or interview. The focus groups and individual interviews were designed and implemented following the generally accepted practices of Morgan et al (1998), Morgan (1997), Morgan (1996), and Weiss (1994) respectively. A Mexican, professional moderator using a specially prepared discussion guide conducted the focus groups and individual interviews. All focus group and individual interviews were tape-recorded and subsequently transcribed.

Data Analysis

The data analysis allowed me to (1) discover themes, (2) consider the choice and meanings of words, (3) consider the context(s) of data collection, and (4) consider the consistency of responses (Krueger 1994). The analysis did not produce simple counts of things, but rather "fractured" the data and rearranged it into categories that facilitated understanding the data and comparing it within and between categories (Maxwell 1996, Strauss and Corbin 1990). The 12 focus groups and 19 individual interview transcripts resulted in more than 500 pages of text. An iterative, grounded theory approach (Strauss and Corbin 1990) was used to code the transcripts. First, almost every word of a randomly selected subset of transcripts was coded (open coding). Next a set of thematic or summary codes was developed (axial coding). When no new open codes were necessary to code additional transcripts, all of the study's transcripts were axial coded. The final iteration of coding of the text (selective coding) focused on organizing the data into 36 categories relevant to respondents' resource use, value, understanding, perception, and control of the ecosystem.

The reported research here focuses on local beneficiaries' use, perception, and understanding of chivita collection in Chelém Lagoon. The data analysis created and used multiple-response variables to record instances that focus-group discussions and individual interviews raised for discussion matters pertaining to chivita (e.g., shellfish collection, sale of chivita, market price for chivita). For example, the variable "Lagoon fishing" captured and documented a wide range of particular "fishing activity" (e.g., mullet, shrimp, crab, chivita, and other species in the lagoon). Use of such variables accommodated the wide range of the discussion topics as well as allowed the coded transcript data to be subsequently analyzed using statistical software. Furthermore, this approach allowed the focus group and individual interview transcript data to be transformed into variables to test research hypotheses and generate value estimates.

RESULTS

The variable, interview type, records the type of interview (e.g., focus group or individual interview) associated with each case of coded data. Other variables capture those fishery services raised by respondents during the focus groups and individual interviews. Table 1 illustrates the four primary fishery services associated by local beneficiaries with the lagoon and that emerged from the focus group and individual interview data. Table 1 also presents some representative references as well as the percentage of focus group and individual interview sessions that raised for discussion each fishery service. As can be seen, participants, in general discussions of the region, the ecosystem, and what they do, talked about "fishing in the lagoon" quite frequently. Interestingly, participants discussed chivita collection most frequently and in almost all of the sessions. The other significant "fishing activities" associated with the lagoon and raised by participants include the collection of crab as bait for use during the limited octopus (*Octopus maya* and *Octopus vulgaris*) fishing season and the increasingly infrequent collection of shrimp in the lagoon. These results are especially noteworthy when one considers that local researchers, regional coastal resource managers, environmentalists, and government officials failed to mention chivita collection as a local

TABLE 1.

Fishery service variables.

Topic variable	Example	% sessions raising topic
Chivita	*Melongena melongena; Melongena corona bispinosa;* used for food and commerce	97
Lagoon fishing	we fish in lagoon; people come to fish in wetland; some use their nets day & night at lagoon entrance	91
Crab	collected as bait; frozen for use during two month octopus season	61
Shrimp	seawater sometimes brings shrimp; when shrimp here, all fish for them; not as many shrimp as in past	39

activity or an ecosystem service in more than 12 interviews conducted prior to the focus groups and individual interviews.

As the data illustrate, the qualitative research revealed information about the use and understanding of the complex coastal ecosystem as a fishery. The qualitative data revealed the importance of chivita collection to the subsistence of the local inhabitants of the Chelém Lagoon region.

Focus groups and qualitative interviews can produce opinion and economic data that is consistent with traditional survey research data (Reynolds and Johnson 1978, Vaughn et al. 1996). The data were also coded to capture respondents'comments on catch rates and prices associated with the collection of chivita. As Table 2 illustrates, respondents reported a range of chivita collection rates and market prices. Some participants mentioned collection rates per working day ranging from as few as 0.5 kilograms to as many as 4 kilograms. Many respondents spoke about once being able to easily collect more than 10 kilograms per day during good times. During the sessions, respondents also volunteered a range of prices being paid for chivita by brokers or middlemen in the region. As Table 2 shows, the average price per kilogram was about 8 pesos or approximately $1.13. The brokers, in turn, transported and sold the chivita to hotels and restaurants in the tourist trade for use as appetizers and table snacks.

It must be pointed out that this area enjoys a potentially lucrative octopus fishing season from mid-August through late-November. While there are reports that chivita and other lagoon species are collected during Octopus season, many men reported focusing on the octopus season, if able. This is not true for the women of the area who reported collecting chivita to sustain their families throughout the year, especially while their husbands go to fish in the sea. As Figure 1 illustrates, the communities of Chelém and Chuburná derive significant economic benefits from their extractive use of chivita from the Chelém Lagoon. Using the median reported catch rates and the median reported market price, it is estimated that chivita collection represents approximately $230,000 to $350,000 dollars of income to these communities. That is, a family relying on the lagoon for chivita collection year-round can generate about $580 dollars annually, or about $390 dollars from chivita if they devote four months completely to octopus fishing. These findings are especially significant when compared to the Mexican minimum wage of 14 pesos ($2) per day paid at factories in the region, when there is available work.

TABLE 2.

Chivita collection reports

Variable	Low	High	Mean
Catch rate (kg/day)	0.5	4	1.8
Price* (Pesos/kg)	5	10	7.9

* Exchange rate at the time about 7 pesos to $1 US.

DISCUSSION

As the foregoing analysis demonstrates, chivita collection is the most significant economic activity associated with the lagoon fishery for the communities of Chelém and Chuburná. While the participants mentioned the collection of small crabs, it turns out that the unidentified species collected is too small to support human subsistence and is used as bait for the brief octopus (*Octopus maya* and *Octopus vulgaris*) season in the nearby Gulf of Mexico. The existing economic difficulties facing these communities and Mexico as a whole repeatedly came to light during the focus groups and individual interviews. The sessions were replete with discussions of the difficulty in providing for one's family. Increasing commercial fishing pressure by trawlers in the offshore and near-shore fisheries of the Gulf of Mexico is reported to have decimated the once rich coastal fishing resource. Local beneficiaries have responded by increasing their reliance upon the lagoon and its mangrove ecosystem for subsistence.

The focus groups and individual interviews left no doubt that lagoon fishing for chivita is of utmost importance to local people. The collection of chivita from the muddy bottom of the Chelém Lagoon has become the predominant subsistence strategy for the regions' communities. Repeatedly, I was told of mothers placing their small children on planks of wood so that they could attend to them while digging for chivita in the lagoon's muddy bottom. Chivita collection has replaced other more conventional lagoon fishing and the collection of crabs as the key lagoon fishery service. When the Gulf of Mexico's fishery was thriving, there were reports that local beneficiaries were using the lagoon only as a place to collect their bait. However, the decline of the near-shore fishery has resulted in changes.

While shrimp collection in the lagoon was mentioned in the sessions, it occurs only occasionally. According to participants, the recent construction of a duck habitat restoration dike by Ducks Unlimited and Mexican Navy activities, have caused drastic curtailment of the once annual or biannual inundation of shrimp in the lagoon. It is reported that these projects have stopped the circular flow of seawater through the lagoon that resulted after Hurricane Gilbert. Another lagoon-based activity that has been curtailed is salt collection. At one time, individuals in the region could construct salt ponds; flood them with seawater, allow the water to evaporate and then collect and sell crystallized sea salt. However, the area's lucrative salt mining business has been defunct for years. This change followed the flooding and ipso facto enlarging of Chelém Lagoon when the Mexican government dredged and constructed a safe harbor and naval station in the lagoon in the late 1960s and early 1970s (Paré and Fraga, 1994).

It seems that virtually every family in the two communities, at one time or another has adopted chivita collection as part of its subsistence survival strategy. Furthermore, it is common for al-

SEASONAL CHIVITA COLLECTION
(Excluding Octopus Season)

8 mo. season x 24 working days/mo. x 1.8 kg/day x 7.9 pesos/kg = 2,733 pesos/household

or

± $ 390* per household

YEAR-ROUND CHIVITA COLLECTION

12 mo. x 24 working days/mo. x 1.8 kg/day x 7.9 pesos/kg = 4,095 pesos/household

or

± $ 585* per household per year

AGGREGATE ANNUAL VALUE TO CHELÉM & CHUBURNÁ
(± 600 households)

Excluding Octopus Season	Year-Round Collection
1,639,800 pesos per year	2,457,000 pesos per year
or	or
± $ 234,257* per year	± $ 351,000* per year

* Exchange rate of 7 pesos per dollar

Figure 1. Benefit of chivita collection.

most everyone in the area to refer to himself or herself as a "pescador" (fisherman) despite the fact that many provide for themselves and their families by working in nearby factories or doing construction work. Individuals when speaking about lagoon fishing perceive themselves as "fisherpeople", and it was learned repeatedly during the sessions that respondents include chivita collection, crab and shrimp collection together with line and net fishing for other species. What makes this especially interesting, is that researchers from nearby Mérida working on coastal zone management in the region were surprised to learn of the extent to which the respondents relied upon chivita collection. It was their belief that chivita was a minor component of residents' subsistence strategy and that near-shore fishing in the gulf was the predominant occupation in the area.

In the words of one respondent:

> We used to make a living fishing in the sea . . . Now you can't make a profit more than 2 to 3 months from fishing in the sea . . . The same problem is also happening in the lagoon, it used to be that you could take all the crab you wanted. Now only the small ones are around . . . While some try to work elsewhere, people sustain their families with chivita from the wetland (Transcript 18).

CONCLUSION

This study demonstrates that chivita collection is not only a benefit to these communities but it is a significant source of their subsistence activity. The data show the value of using individual interviews and focus groups to learn from local beneficiaries about their ecosystem and natural resource use. The finding that the West Indian crown conch (*Melongena melongena* L.), *Caracol Negro* [Black conch] (*Melongena corona bispinosa*), and the Florida crown conch (*Melongena corona*) are significant sources of subsistence and income, place these species in a new economic light. For the people of Chelém Lagoon, these species are welcome visitors not predators.

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YELLOW AND BROWN SHELL COLOR MORPHS OF *CORBICULA FLUMINEA* (BIVALVIA: CORBICULIDAE) FROM SICHUAN PROVINCE, CHINA, ARE TRIPLOIDS AND TETRAPLOIDS

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ABSTRACT Yellow and brown shell color morphs were distinguished in samples of *Corbicula fluminea* collected from Anyue County, Sichuan Province, China. Shells of yellow morphs are straw yellow externally and white internally, while those of brown morphs are dark brown and purple, respectively. Karyological and DNA microfluorometric analyses revealed that yellow and brown morphs are triploid and tetraploid, respectively. Both are simultaneous hermaphrodites with non-reductional and biflagellate spermatozoa and brood their larvae in the inner demibranchs like diploid *C. fluminea* in Taiwan and Japan. These reproductive characteristics are similar to triploid *Corbicula leana* in Japan, which is self-fertilizing. These results suggest that *Corbicula fluminea* at different ploidy levels may also reproduce by self-fertilization.

KEY WORDS: *Corbicula fluminea*, triploid, tetraploid, karyotype, DNA microfluorometry

INTRODUCTION

The freshwater bivalves, *Corbicula leana* (Müller) and *Corbicula fluminea* (Müller), are hermaphroditic and brood their larvae (Miyazaki 1936, Kraemer and Galloway 1986). In both species, one individual can reproduce by self-fertilization (Ikematsu and Yamane 1977, Kraemer 1978). Both species show little allozyme polymorphism (Hillis and Patton 1981, Sakai et al. 1994). This monogenic condition has been explained as self-fertilization (Mcleod and Sailstad 1980) or gynogenetic development (Okamoto and Arimoto 1986).

C. leana in Japan is triploid and *C. fluminea* in Taiwan and Japan is diploid, both species producing non-reductional spermatozoa (Okamoto and Arimoto 1986, Komaru et al. 1997). We have also produced cytological evidence of spontaneous androgenetic development in *C. leana* (Komaru et al. 1998). These similar reproductive characters suggest that diploid *C. fluminea* may also reproduce by self-fertilization.

In this study, we identified two obviously different shell color morphs of *C. fluminea* and studied their chromosomes, spermatozoa, and somatic cell DNA to identify genetic differences between and reproductive modes in them.

MATERIALS AND METHODS

The Samples

The *Corbicula fluminea* (Müller) samples were collected from a stream in Anyue County, Sichuan Province, People's Republic of China, in September 1997. The two color morphs were collected at the same sampling site. The samples were transferred to Sichuan University, Chengdu, Sichuan, China and the National Research Institute of Aquaculture, Nansei, Mie, Japan, and kept in laboratory aquaria.

Chromosomes

The samples were transferred to freshwater containing 0.002% colchicine for 4–5 hours. The gills and gonads were then dissected out and hypotonically treated with distilled water for 30 min and fixed in Carnoy's fixative. Small pieces of gill or gonad were minced in 50% acetic acid and isolated cells were placed on slides, air-dried, and stained with 2% Giemsa in phosphate buffer (pH = 7.2) (Okamoto and Arimoto 1986). For chromosome classification and karyotyping, metaphase chromosomes were measured and paired on the basis of relative length and centromeric index.

DNA Microfluorometry

Spermatozoa and somatic cells from the mantle were isolated in distilled water, placed on slides, air-dried, and fixed with 70% ethanol. The slides were then stained with a DAPI staining solution. Relative DNA content was estimated by microfluorometry after DAPI staining (Komaru et al. 1988).

Light Microscopy

Gonads and gills were fixed with 10% formaldehyde and processed for paraffin sectioning. Slides were stained with Mayer's hemalum and eosin.

Spermatozoa Measurement

Spermatozoa were isolated from the gonads in freshwater and observed by phase contrast microscopy. The lengths of the sperm head and flagella were estimated using an ocular micrometer.

RESULTS

Shell Color and Size

Figure 1 shows the two shell color morphs (yellow and brown) of *Corbicula fluminea* from Anyue County. Externally, the shell surface of the brown morph is dark brown (Fig. 1A), and its inner surface is deep purple along the ventral margin, but rather white from the pallial line to the umbo (Fig. 1B). The external surface of the yellow morph is straw-colored (Fig. 1A) while the inner surface is white (Fig. 1B). This form is also characterized by purple flashes along the anterior and posterior lateral teeth.

Shell lengths (SL), heights (SH), and widths (SW) were not significantly different between the yellow and brown morphs, but the three ratios of SW/SL, SW/SH, and SW/ (SL+SH+SW) are significantly larger in the yellow than in the brown morph, whereas the ratio of SH/SL in both morphs is similar (Table 1).

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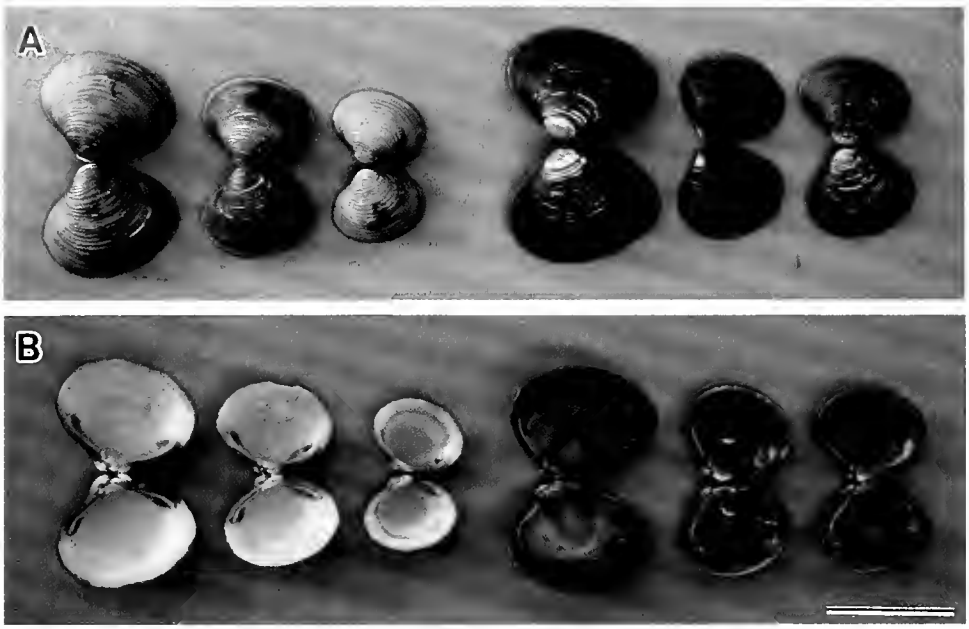

Figure 1. External (A) and internal (B) views of the shells of yellow (left) and brown (right) morphs of *Corbicula fluminea* collected from Anyue County, PRC.

Chromosomes and Relative DNA Content of Somatic Cells

Chromosome numbers of the yellow morph were 54 in the gills (Fig. 2A) and 18 in the gonads (Fig. 2B), and 72 in the gills of the brown morph (Fig. 2C). Eight well-spread mitotic metaphases of the yellow morph and seven of the brown were measured and karyotyped. Figure 3 shows the karyotype taken from one metaphase of the yellow morph containing 18 triplets arranged on the basis of shared relative lengths and centromeric indices. It consists of 2 metacentrics, 13 submetacentrics (including 3 submetacentrics-metacentrics and 6 submetacentrics-subtelocentrics), and 3 subtelocentrics (Table 2). Figure 4 shows the karyotype of the brown morph. It contains 18 quadraplets, consisting of 2 metacentrics, 13 subcentrics (including 3 submetacentrics-metacentrics and 6 submetacentrics-subtelocentrics), and 3 subtelocentrics (including 2 subtelocentrics-submetacentrics) (Table 3). The meiotic chromosomes of the yellow morph have triplet-like chromosomes and an incomplete synapsis of homologous chromosomes was observed (Fig. 2B). Meiotic chromosomes could not be observed, however, in any studied individuals of the brown morph.

The relative DNA content of the yellow morph was almost the same as that of the triploid *C. leana* collected from Mie Prefecture, Japan, whereas that of the brown morph was about 1.3 times that of triploid *C. leana* (Table 4).

DNA Content of Sperm and Somatic Cells

As shown by DNA microfluorometry of the spermatozoa and somatic cells (Table 5), the relative DNA content of the former was almost identical to that of the latter. Seven yellow morph and eight brown morph individuals all produced spermatozoa with a DNA content similar to that of their own somatic cells.

Spermatozoa Measurements

The sperm heads of both *C. fluminea* morphs are slightly curved and elongated and both have two flagella. The length of the brown morph spermatozoa is significantly longer than that of the yellow. The sperm head size was 20.3 ± 1.0 μm (n = 10) for the yellow morph and 24.0 ± 0.5 μm (n = 10) for the brown morph. Flagella length was 46.2 ± 1.5 μm (n = 10) in the yellow morph and 49.5 ± 1.9 μm (n = 10) in the brown one.

Gonad and Larval Incubation

Gonad and gill sections of 14 yellow and 12 brown morphs were examined. All individuals were simultaneous hermaphrodites. Three of the 14 yellow morph individuals and four of the 12 brown morph were brooding larvae in their inner demibranchs.

TABLE 1.

A comparison of shell dimension ratios between yellow and brown morphs of *Corbicula fluminea*.

	SH/SL	SW/SL	SW/SH	SW/(SL + SW + SH)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Y morph	0.822 ± 0.022	0.567 ± 0.016	0.690 ± 0.016	0.238 ± 0.007
B morph	0.847 ± 0.021	0.478 ± 0.020	0.564 ± 0.024	0.206 ± 0.006

Y: yellow morph of *C. fluminea*
B: brown morph of *C. fluminea*

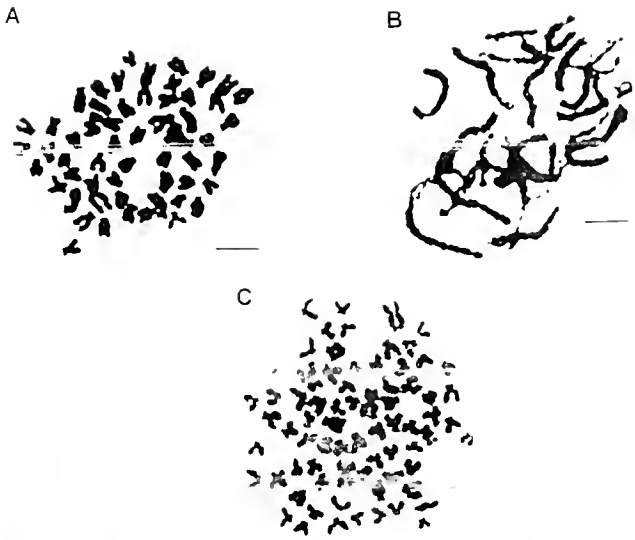

Figure 2. Mitotic and meiotic chromosomes of yellow (A, B) and brown (C) morphs of *C. fluminea*. Scale bar = 5 μm.

DISCUSSION

C. fluminea from Japan and Taiwan have been reported to be hermaphroditic and diploid with $2n = 36$ in the gills and 18 bivalents in the gonads (Komaru et al. 1997). In the present study, yellow and brown color morphs of *C. fluminea* were discovered in Anyue County, Sichuan Province, China, where they are sympatric. According to our karyological and DNA content analyses of these two morphs of *C. fluminea*, the yellow one is triploid with $3n = 54$ in the gill tissues, which can be classified into 18 triplets on the basis of their relative lengths and centromeric indices, and with 18 trivalents in the gonads. The brown morph is tetraploid with $4n = 72$ in the gill tissues, which can be classified into 18 tetraplets.

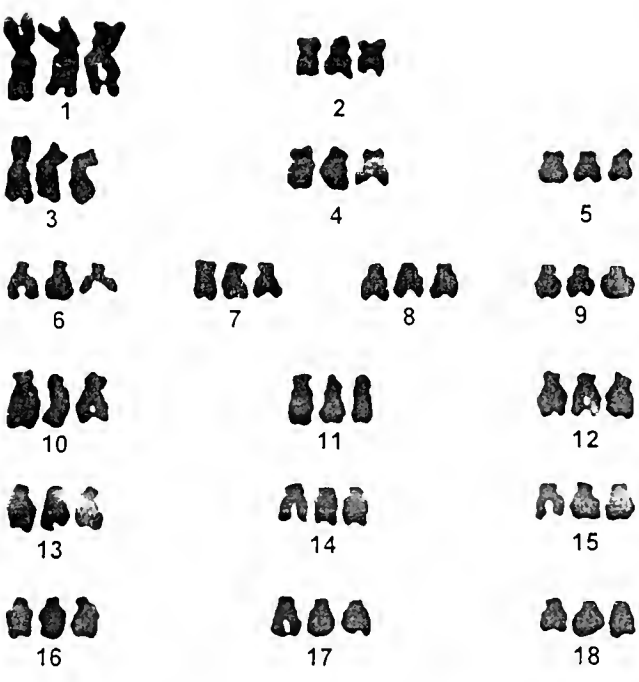

Figure 3. Karyotypes taken from yellow ($3n = 54$) morph of *C. fluminea* metaphases. Scale bar = 5 μm.

A comparison of karyotypes from triploid and tetraploid *C. fluminea* shows that they have a similar formula containing 2 metacentrics, 13 submetacentrics, and 3 subtelocentrics. This implies that they may have the same origin. However, there are also some differences between the karyotypes, so that the No.2 chromosomes of both the triploid and tetraploid had submetacentrics of different relative lengths. These differences indicate that they may have different genetic properties.

The shell color of *C. fluminea* is highly polymorphic (Morton 1987, Chen et al. 1995). Britton and Morton (1986) also identified two different color morphs of *C. fluminea* in USA with significantly different mean shell dimension ratios, and suggested that such polymorphism in shell color and shell size were the result of environmental induction. Morton (1987) identified the same two morphs in Hong Kong and Tsoi et al. (1991) showed there was no genetic differentiation between the two morphs by allozyme analysis. However, triploid and tetraploid *C. fluminea* with different shell colors and sizes in Anyue County are possibly sympatric, which indicate that these distinct differences in the two shell color morphs may be mainly derived from their different genetic constitutions. Physiological studies should be implemented to determine the fitness of both morphs and the significance of the polyploidy.

Triploid and tetraploid *C. fluminea* are hermaphrodites and brood their larvae in the inner demibranchs. The meiotic chromosomes comprise 18 trivalents with an incomplete synapsis of homologous chromosomes in the gonads of triploid *C. fluminea*. These could not be observed in tetraploid *C. fluminea*. These results imply that the triploid *C. fluminea* cannot carry out normal meiosis as in the triploid *C. leana*, and tetraploid *C. fluminea* fail to achieve meiosis. DNA microfluorometry of spermatozoa and somatic cells of the triploid and tetraploid *C. fluminea* showed that they all produce non-reductional and biflagellate spermatozoa similar to *C. leana* in Japan and *C. fluminea* in Taiwan and Japan (Komaru et al. 1997). These results indicate that these polyploids can reproduce in ways different from the normal sexual method. It has been reported that triploid *C. leana* in Japan reproduces by androgenesis which Komaru et al. (1998) cytologically confirmed. In androgenesis, all maternal chromosomes were expelled as polar bodies at the first division after self-fertilizaton, which results in the forming of only one male pronucleus from the spermatozoa but no female pronucleus. Therefore, only the chromosomes deriving from the male pronucleus formed the mitotic chromosomes for the first zygotic cleavage. Diploid (Komaru et al. 1997), triploid, and tetraploid *C. fluminea* have the same reproductive characters as *C. leana*, i.e., hermaphroditic, brooding, and the production of non-reductional spermatozoa. It is possible that *C. fluminea* with different ploidies may also reproduce by androgenesis and be fertilized by autosperm as in *C. leana*.

In the animal kingdom, polyploids are rather rare, as compared with plants. Animals reproducing by parthenogenesis are sometimes polyploid (Hughes 1989). Three different ploidy levels have been known in *Corbicula* up to now. How did they evolve from an ancestral species? We have shown that diploid and triploid *Corbicula* produce non-reductional spermatozoa (Komaru et al. 1997). These data suggest that diploid androgenetic individuals may evolve from a diploid ancestral population with normal meiosis. After androgenesis arose in an ancestral population, mutations for

TABLE 2.

Measurements and classification of metaphase chromosomes taken from the yellow morph of *Corbicula fluminea*.

Chromosome pair no.	Relative length		Arm ratio		Centromeric index		Classification
	Mean	SD	Mean	SD	Mean	SD	
1	10.87	0.23	1.385	0.126	42.05	2.28	m
2	4.66	0.25	1.339	0.023	42.82	0.44	m
3	7.89	0.42	1.904	0.478	34.97	5.96	sm-m
4	5.55	0.38	1.899	0.200	36.06	1.15	sm-m
5	3.93	0.20	1.724	0.027	36.84	0.33	sm-m
6	4.72	0.23	2.117	0.077	32.45	0.42	sm
7	4.73	0.12	2.187	0.087	31.48	0.86	sm
8	4.61	0.11	2.098	0.046	32.28	0.47	sm
9	4.10	0.16	2.402	0.163	29.58	1.43	sm
10	7.41	0.66	2.494	0.611	28.77	4.87	sm-st
11	6.29	0.71	2.413	0.340	28.85	1.99	sm-st
12	5.74	0.18	3.014	0.038	25.15	0.36	sm-st
13	5.61	0.28	2.781	0.041	27.19	0.75	sm-st
14	4.93	0.09	2.690	0.056	27.13	0.26	sm-st
15	4.43	0.11	2.902	0.093	25.99	0.43	sm-st
16	5.15	0.14	3.488	0.087	22.54	0.54	st
17	4.76	0.23	3.536	0.064	22.12	0.47	st
18	4.46	0.13	3.578	0.042	22.37	0.38	st

polyploidy occurred. Lokki (1976) pointed out that in parthenogenetic animals, polyploidy would help to maintain functional loci when mutant alleles are accumulating. This could explain polyploidy in *Corbicula*. It is likely that the triploids and tetraploids in *Corbicula* have arisen from diploids. To increase the ploidy, we assume two possible androgenetic events in *Corbicula*: either (1), production of spermatozoa with increased ploidy levels derived from meiotic failure during spermatogenesis or (2), incomplete polar body formation, i.e., one or two maternal chromosome sets were not extruded and participate in pronucleus formation.

The taxonomy of *Corbicula* has problems (Morton 1986) because the genus contains species and populations that can repro-

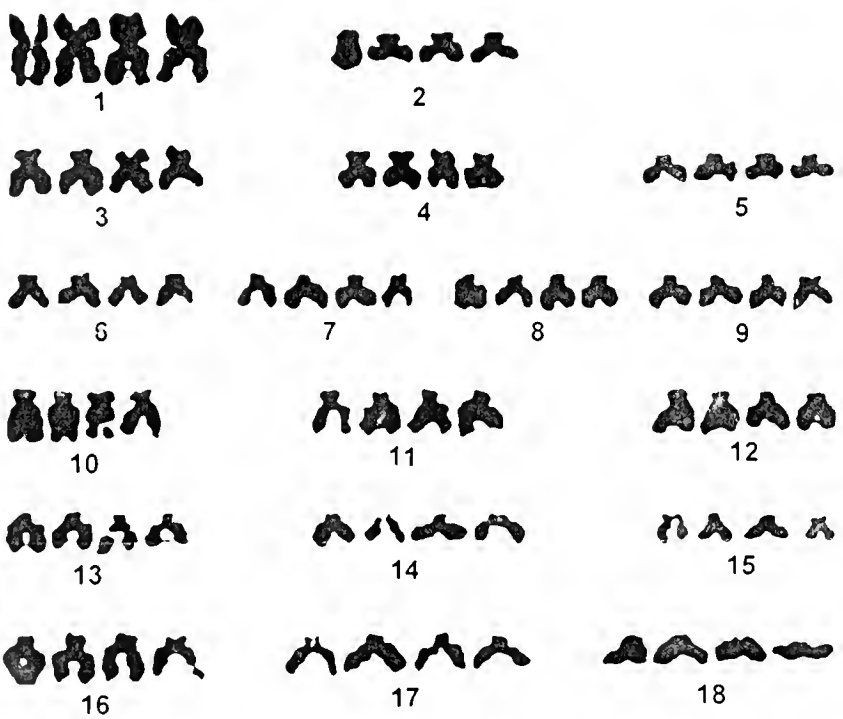

Figure 4. Karyotypes taken from brown (4n = 72) morph of *C. fluminea* metaphases. Scale bar = 5 μm.

TABLE 3.

Measurements and classification of metaphase chromosomes taken from the brown morph of *Corbicula fluminea*.

Chromosome pair no.	Relative length		Arm ratio		Centromeric index		Classification
	Mean	SD	Mean	SD	Mean	SD	
1	9.96	0.22	1.268	0.069	44.09	1.29	m
2	4.87	0.04	1.796	0.060	37.08	0.48	m
3	6.33	0.08	1.806	0.029	35.59	0.39	sm-m
4	5.07	0.11	1.728	0.041	36.67	0.45	sm-m
5	4.18	0.06	1.923	0.059	34.25	0.71	sm-m
6	4.88	0.07	2.198	0.034	31.79	0.56	sm
7	4.97	0.03	2.268	0.025	30.58	0.23	sm
8	4.80	0.05	1.975	0.023	33.74	0.18	sm
9	4.68	0.03	1.960	0.017	33.75	0.19	sm
10	6.95	0.11	2.487	0.021	28.68	0.34	sm-st
11	6.42	0.16	2.597	0.092	27.86	0.47	sm-st
12	5.71	0.10	2.689	0.044	27.24	0.32	sm-st
13	5.02	0.08	2.818	0.083	26.21	0.27	sm-st
14	4.48	0.06	2.734	0.055	26.83	0.54	sm-st
15	4.07	0.12	2.742	0.066	26.84	0.48	sm-st
16	7.22	0.09	3.049	0.048	24.78	0.56	st-sm
17	5.90	0.07	3.042	0.036	24.78	0.44	st-sm
18	4.22	0.05	4.152	0.029	19.51	0.41	st

duce by self-fertilization and androgenesis. The taxonomy of *Corbicula* should, thus, be determined on the basis of not only shell morphology but also genetic and ecological information. The key to *Corbicula* taxonomy is how species and populations reproduce and how they evolved from an ancestral diploid species. Further studies on the genetic differences and reproductive modes of both morphs of *Corbicula fluminea* are required to identify the evolutionary significance of androgenesis and polyploidy.

Interestingly, there are a few brown individuals with almost the same DNA content as yellow ones, such as B-2 and B-8 in Table 5. It is unknown whether they belong to another polymorphic type of *C. fluminea*, but it is worth studying further. Maybe the genetic polymorphism of *C. fluminea* is much more intricate than what we know until now.

TABLE 4.

Ploidy estimation by DNA microfluorometry of yellow (Y1-10) and brown morphs (B1-11) of *C. fluminea* collected from Anyue County.

No.	Mean (S.D.) of sample[a]	Mean (S.D.) of 3n standard[b]	Ratio[c]	Plody
Y-1	376.76 (11.08)	369.95 (11.08)	1.01	3n
Y-2	341.78 (15.06)		0.92	3n
Y-3	381.26 (11.56)		1.03	3n
Y-4	352.75 (12.01)	377.85 (14.97)	0.93	3n
Y-5	378.23 (9.24)		1.00	3n
Y-6	315.68 (14.10)	344.65 (5.48)	0.92	3n
Y-7	310.63 (12.50)		0.90	3n
Y-8	337.37 (18.56)		0.98	3n
Y-9	347.40 (12.00)	355.60 (13.90)	0.98	3n
Y-10	350.45 (10.10)		0.99	3n
B-1	478.37 (11.96)	353.60 (9.50)	1.35	4n
B-2	488.20 (11.83)		1.38	4n
B-3	497.37 (22.49)	356.62 (14.93)	1.37	4n
B-4	484.89 (10.92)		1.36	4n
B-5	459.39 (22.62)		1.29	4n
B-6	454.56 (15.31)	344.07 (12.60)	1.32	4n
B-7	468.14 (12.34)		1.36	4n
B-8	484.95 (12.09)	375.08 (10.90)	1.29	4n
B-9	478.75 (20.00)		1.28	4n
B-10	477.95 (11.96)	352.09 (8.03)	1.36	4n
B-11	484.12 (11.83)		1.37	4n

[a] Mean of Relative DNA content (fluoroscence intensity) of at least 20 cells at each sample.
[b] Triploid *Corbicula leana* collected from Mie Prefecture, Japan.
[c] Ratio = Mean of sample/Mean of 3n standard.

TABLE 5.

Mean and standard deviation of relative DNA content of spermatozoa and gill cells in yellow (Y1-7) and brown morphs (B1-8) of *C. fluminea* collected from Anyue County, People's Republic of China by microfluorometry.

Shell color morph	DNA content	
	Sperm	Somatic cell
Y-1	257.48 ± 12.58 (n = 40)	262.50 ± 20.65 (n = 33)
Y-2	293.71 ± 27.43 (n = 49)	314.41 ± 23.76 (n = 53)
Y-3	276.81 ± 10.68 (n = 48)	299.23 ± 16.05 (n = 52)
Y-4	257.48 ± 12.58 (n = 33)	262.50 ± 20.05 (n = 40)
Y-5	286.63 ± 15.08 (n = 60)	284.48 ± 23.08 (n = 60)
Y-6	293.71 ± 27.43 (n = 49)	314.41 ± 23.76 (n = 49)
Y-7	257.48 ± 12.58 (n = 33)	262.50 ± 20.05 (n = 40)
B-1	405.43 ± 25.01 (n = 58)	443.38 ± 31.99 (n = 58)
B-2	316.23 ± 16.90 (n = 47)	299.00 ± 19.22 (n = 42)
B-3	333.31 ± 23.61 (n = 55)	357.62 ± 35.00 (n = 45)
B-4	358.38 ± 13.13 (n = 50)	362.23 ± 16.93 (n = 50)
B-5	372.36 ± 23.71 (n = 52)	405.94 ± 24.36 (n = 51)
B-6	405.43 ± 25.01 (n = 58)	443.38 ± 31.99 (n = 59)
B-7	358.93 ± 24.56 (n = 66)	349.24 ± 20.93 (n = 65)
B-8	247.00 ± 17.65 (n = 31)	249.44 ± 21.81 (n = 34)

The numbers in parenthesis are the number of cells estimated.

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LACK OF SURFACE-ASSOCIATED MICROORGANISMS IN A MIXED SPECIES COMMUNITY OF FRESHWATER UNIONIDAE†

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ABSTRACT To determine whether unionids contain surface-attached endosymbiotic bacteria, ciliates, or fungi, we used scanning electron microscopy to examine the epithelial surface of various organs within the digestive system and mantle cavity of temperate river and lake unionids on a seasonal basis. We also cultured material removed from the lumen of these same organs and from the mantle cavity to detect cellobiose-, cellulose- and chitin- degrading microbes. No true endosymbiotic fauna were observed attached to the surface of the digestive or mantle tissues of any species of unionid. Microbial growth on cellulose or chitin bacteriological media varied by season and habitat, but not by unionid species or source of the isolate. Lake unionids did not contain microbes capable of digesting cellulose or chitin, whereas unionids from the river site did in March and August, but not in December. Since these cultured cellulose- and chitin-degrading bacteria were never found attached to any unionid tissues, they appeared to be transient forms, not true endosymbionts. Microbes capable of digesting cellobiose were found in every unionid collected in all seasons and habitats, but again, no microbes were directly observed attached to unionid tissues. If unionids, like most other invertebrates, lack digestive enzymes required to initiate primary bond breakage, then the lack of cellulolytic and chitinolytic endosymbionts would affect the ability to utilize cellulose or chitin foods. Thus, in captivity dry feeds based on corn, soybeans, or nauplii should be pre-digested to ensure maximum absorption of nutrients by unionids. The lack of cellulolytic or chitinolytic endosymbionts should not affect relocation success, though the seasonal role of transient microbes in unionid nutrition requires further investigation.

KEY WORDS: gut microflora, endemic microbes, Unionidae

INTRODUCTION

Freshwater unionids in North America are being extirpated at alarming rates due to factors such as habitat degradation and competition from the exotic zebra mussel (*Dreissena polymorpha* Pallas) (Williams et al. 1993). At this time, conservation efforts for adult unionids include relocation into new habitats and intensive aquaculture, but mortality rates have been high with most adults surviving less than three years (Cope & Waller 1995; Lellis and Johnson 1998; Gatenby et al. 1999). Although these high mortality rates are undoubtedly due to a host of different factors, one problem area has been identification of dietary requirements. Little information exists on dietary preferences and required nutrients necessary for formulating a captive diet, and evaluating food resources in new habitats or refugia. The few dietary studies in natural habitats have shown that unionids ingest a wide variety of potential food items including algae, detritus, fungus, rotifers, and zooplankton, with detritus as the dominant component in both the mantle cavity and gut lumen (Lefevre & Curtis 1910; Jiffry 1984; McMahon 1991; Nichols & Garling 2000).

The ingestion of large amounts of detritus implies that detritus is actively selected and thus may play a significant dietary role. This role is not easy to characterize as detritus in aquatic systems represents a complex matrix often containing cellulose from aquatic and terrestrial sources, chitin from fungus, rotifers, and zooplankton, and which has in turn been colonized by bacterial, fungal, and protistan fauna. Thus, ingested detritus could function merely as a substrate for the preferred food item, the associated epiorganisms, or could function as a direct source of nutrients obtained from cellulose or chitin degradation. One complicating factor is that cellulose and chitin are highly complex polysaccharides that require specific enzymes to initiate primary bond breakage. Such primary enzymes are rarely produced endogenously in aquatic invertebrates, and are recorded for very few marine bivalves, although cellulases in general have been detected in both marine and freshwater mollusks. In unionids, the concentration of cellulases present has actually proven to be a successful technique for assessing the health status of the animal (Haag et al. 1993; Farris et al. 1994). However, these cellulase concentrations were obtained from total animal bioassays, from unionids freshly removed from the field, and were not further identified to type. It has not been determined if primary cellulases were present, and if so, whether they were produced endogenously by the unionid, or exogenously by the microbial community associated with ingested food items. Furthermore, chitinases of any type have not yet been reported for either freshwater or marine bivalves.

Most aquatic animals that feed directly on detrital cellulose access the necessary primary cellulases through a symbiotic relationship with some type of bacteria/microbe, and contain recognizable endosymbiotic fauna somewhere in their digestive tracts (black fly larvae, Taylor et al. 1995; crane fly larvae, Klug & Kotarski 1980; mullet, Mountfort & Rhodes 1991). Microbial and endosymbiont/nutritional relationships among bivalves are far more complex than those reported for other aquatic invertebrates and often vary by species as well as by location. True endosymbiotic relationships range from the consistent obligate communities of microbes directly buried within gill tissues of deep-sea hydrothermal vent bivalves (Wood & Kelly 1989), to spirochaetes merely attached to the outside epithelial layer of digestive tissue or to the crystalline style (Bernard 1970; Conway & Capuzzo 1989; Prieur et al. 1990). Geographical variability is common, with endosymbionts existing inside some species at some locations, but not in others (Bernard 1970), while some marine bivalves never exhibit endosymbiotic relationships (Garland et al. 1982). Studies on freshwater bivalve- microbial relationships are very limited (Starliper et al. 1997) and have focused mainly on bacterial communities found in the gut lumen or passing through the intestinal tract. As in marine bivalves (Prieur et al. 1990; Harris et al. 1998),

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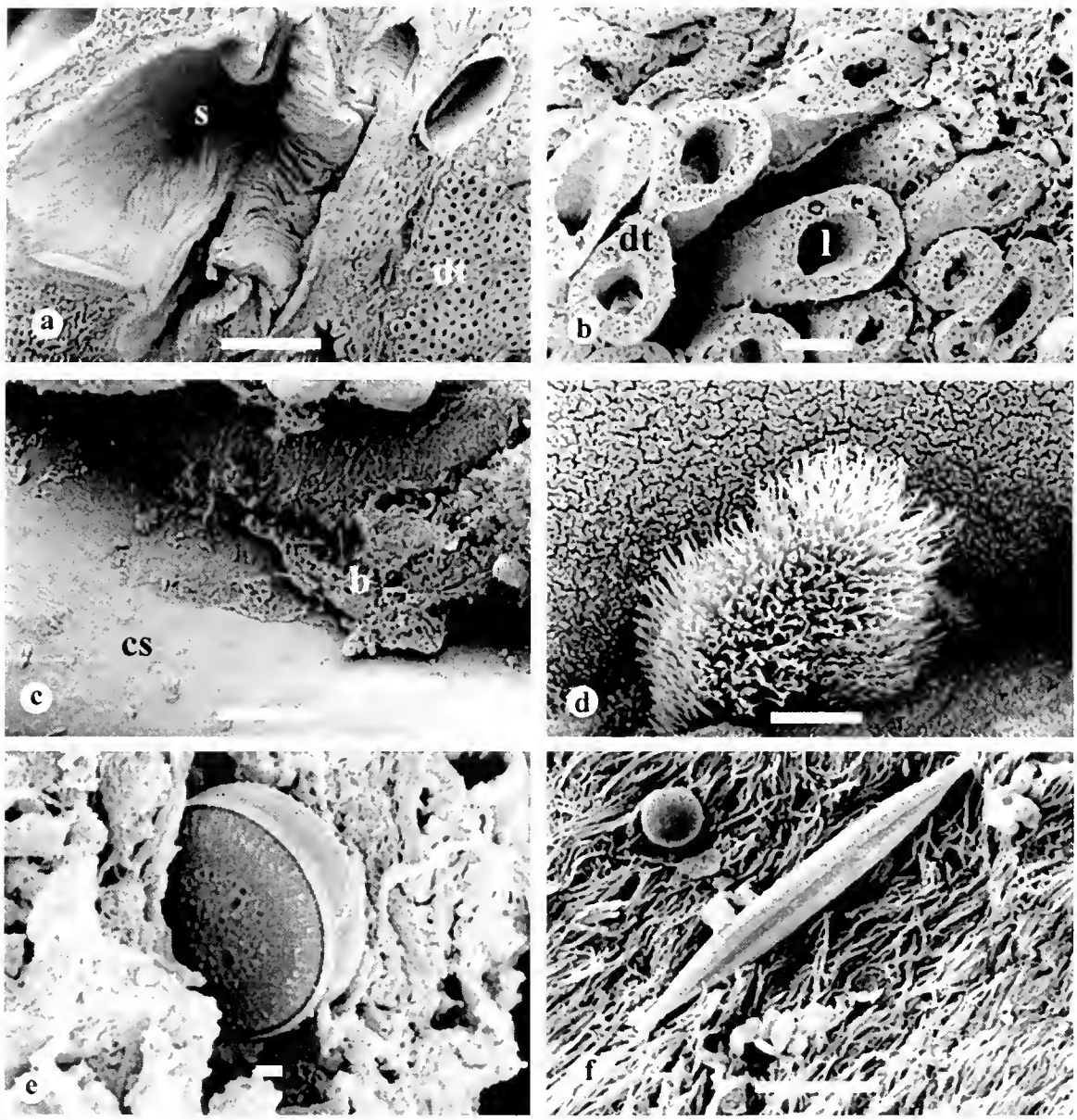

Figure 1. Scanning electron micrograph of unionid digestive tissue examined for the presence of endosymbionts. a = Cross-section of the stomach (s), intestine (i), and digestive gland tubules (dt); b = digestive gland tubules and lumen (l) with food particles; c = surface of crystalline style (cs) showing mucous sheet and food bolus (b); (d) ciliary tuft found in the intestinal tract; (e) and (f) mucous sheet and food material (diatoms and detritus) on the surface of the intestinal tract. Scale bars = 1mm (a); 100μm (b and e); 10 μm (d and f); 1μm (e).

bacterial communities passing through the gut of freshwater unionids usually differ in species composition from those found in the surrounding water column, reflecting selective predation and subsequent enhancement of bacteria species not directly consumed. However, these are not necessarily residential, or endosymbiotic, populations.

The objective of our study was to determine if certain unionid species contained endosymbiotic microflora that might aid in the digestion of dietary materials such as cellulose and chitin, and if present, did this flora vary by location, season, or species of unionid.

MATERIALS AND METHODS

Study Sites and Unionids

The unionids used in this survey were collected from three sites in southeast Michigan, U.S.A.: the Huron River, Four Mile Lake, and Vineyard Lake. The Huron River is a regulated stream, consisting of a series of impoundments connected by free-flowing stretches of river. Our study area focused on the middle section of the river, where the water is shallow (1 m deep), with an average water velocity of 0.5 m/sec and heavy canopy cover dominated by deciduous trees (Livingston Co., 42°25′41″N, 83°54′40″W). A total of 17 unionid species occur in this river. Four were selected for analysis: *Lampsilis ventricosa* Barnes, *L. siliquoidea* Barnes, *Ptychobranchus fasciolaris* Rafinesque, and *Pyganodon grandis* Say. Four Mile Lake is a 25 hectare lake once used as a marl mining site (Washtenaw Co., 42°20′16″N, 83°58′14″W). Except for the marl pit, the lake is shallow, <2 m deep, with no current flow, and a soft substrate covered with *Chara* spp. Canopy cover is lacking, although some wetland vegetation occurs at the edges of the lake. Only one species, *P. grandis*, regularly occurs in this lake. Vineyard Lake is only 12 hectares is size, is 6.5 m deep, and is sur-

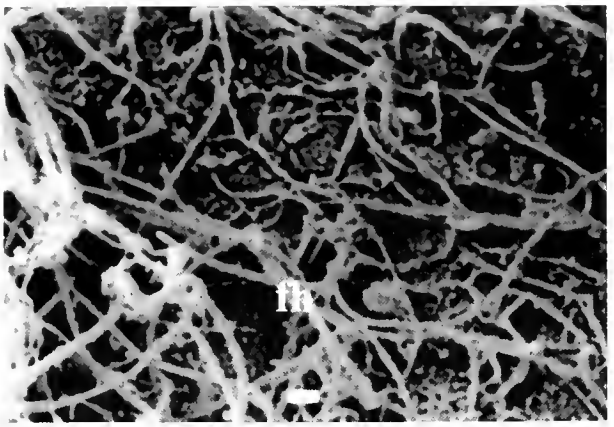

Figure 2. Scanning electron micrograph of fungal hyphae (fh) found growing on the mantle tissue of a single specimen of *Lampsilis siliquoidea* collected from the Huron River in August. Scale bar = 1μm.

rounded by subdivisions (Jackson Co., 42°04′57″N, 84°12′35″W). Only one species, *Elliptio dilatata* Rafinesque, was observed in this lake.

Examination for Surface-Attached Microbes

Sampling for unionids occurred in March, August, and December, and 4 individuals of each species were collected from the Huron River (four species), Four Mile Lake (one species), and Vineyard Lake (one species) (total $n = 24$ unionid specimens for each month for all three sampling locations). Unionids were placed in damp mesh bags in a cooler, and transported to the laboratory. The shell exterior of each unionid was then scrubbed with a brush to remove external flora, rinsed in distilled water, followed by a 10% acetic acid rinse, a second distilled water rinse, and then the shell was pried open. The mantle cavity was rinsed for 3–5 minutes with distilled water and all the rinse water and flushed material was saved. The foot, gills, labial palps, siphons from the mantle cavity, stomach, digestive gland, style sac, style, and fore-mid-hind sections of the intestinal tract were excised and processed for scanning electron microscopy (SEM). Distilled water used to rinse and flush contents from the lumen of each part of the digestive tract with the contents from each organ, as well as the material rinsed from the mantle cavity, were saved separately for culture on bacteriological media.

For scanning electron microscopy (SEM), the separate organs and tissues removed from each unionid were immersed in 10% neutral buffered formalin (primary fixation). After the tissue firmed, serial sections of approximately 1 mm were taken using a scalpel. The tissue was then isolated if needed, trimmed, and washed three times for 20 minutes each in cacodylate-HCl buffer at a pH of 7.2. Secondary fixation involved immersion in 1% cacodylate-HCl buffered osmium tetroxide for one hour. Samples were then dehydrated in a graded alcohol series: 50% (15 min), 65% (15 min), 75% (15 min), 85% (15 min), 95% (15 min), 100% (3 changes 15 min each). Tissues were then critical point dried in CO_2 transition fluid, mounted on 2.5 cm stubs, sputter coated with gold, and examined under a scanning electron microscope.

Microbial Growth on Bacteriological Media

The contents and rinse water were removed from seven areas within each unionid: mantle cavity, stomach, digestive gland, style sac, fore-, mid-, and hind gut. These samples from each of the four individual animals of a particular species collected at each locality were pooled and then used to inoculate three standard enrichment broths, cellulose (carboxymethylcellulose), chitin, or cellobiose. The same amount of inoculant (about 0.10 cc), was removed from each well-stirred pooled sample and placed into a test tube containing cellulose, chitin, or cellobiose with two duplicate sets made (7 internal sources of the inoculant × 2 replicates × 3 media types = 42 total samples per sampling date). This yielded a total of 168 samples for all species in the Huron River each sampling date, and 42 for Vineyard and Four Mile lakes since each had one unionid species present. The duplicate samples for each species and site were randomly split into two groups (3 types of culture media from 7 tissue locations listed above) and then placed in anaerobic or aerobic conditions at room temperature (24°C) using techniques described in Bryant and Burkey (1953, substitute chitin for cellobiose), Bryant (1972), and Hungate (1950). Anaerobic conditions were maintained under a gas phase of 100% CO_2 without agitation. Culture tubes were examined for the presence of fermentation end products (gas production) and media degradation (clouding of media and breakdown of larger chitin particles) after 12, 24, 48, 72, 96, and 120 hours.

RESULTS

Examination for Surface-Attached Microbes

Examination of tissues using the scanning electron microscope showed no attached bacteria, fungi, microciliates, or other types of

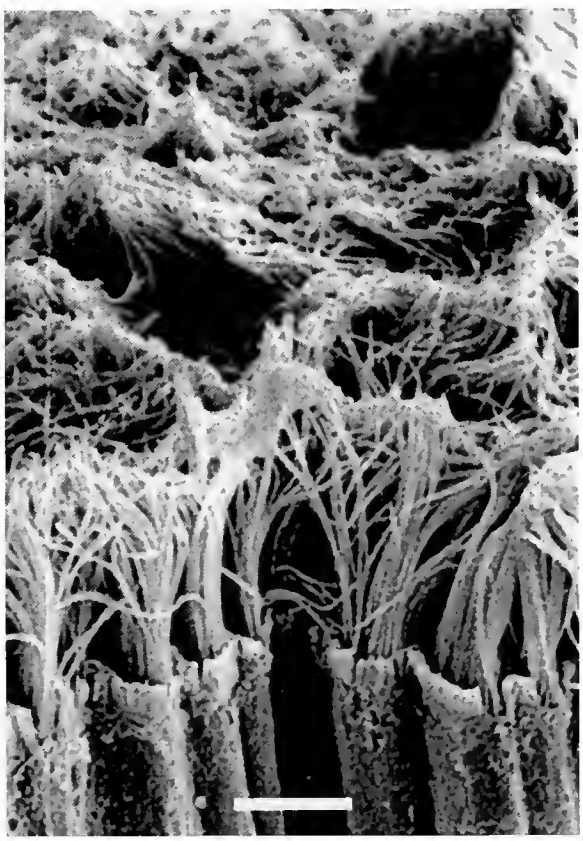

Figure 3. Scanning electron micrograph of unionid intestinal tract showing ciliated enterocytes and the loose junctions between the cells. Note, no obvious mass of endosymbiotic microflora can be observed. Scale bar = 10 μm.

microbes found on the epithelial surface of various mantle and digestive organs (foot, gills, labial palps, stomach, ciliary tufts, crystalline style and style sac, digestive gland, and fore-mid-hind intestinal tract) except in one animal (Fig. 1a-d). All epithelial layers of these tissues were covered in March and August with a heavy layer of mucus. Food particles, including bacteria, were often seen trapped in this mucus layer (Fig. 1e and f), but no attached bacteria or other microbes were associated with this mucus layer or below it on the surface of the epithelium itself. The one exception was a fungal mat growing on the mantle tissue, at the base of the excurrent siphon, collected from one *L. siliquoidea* in August (Fig. 2).

Even though no obvious attached microbes were seen, every unionid examined, from all sites, contained what appear to be attached round structures, possibly spores, in their intestinal tracts. These structures were always found below the mucus layer and between the enterocytes comprising the intestinal wall (Figs. 3 and 4). They were large, about 1μ in diameter, and were attached by a stalk to the unionid tissue (Fig. 4d and f). Though consistently seen, attempts to rear these spores (if that is what they were) in isolation, for further elucidation, failed.

Microbial Growth on Bacteriological Media

Microbial growth varied by locality, media type, and season but not by unionid species, replicate, organ source, or the presence or absence of oxygen. All (100% of the 336 samples) of the samples from the Huron River population collected in March and August

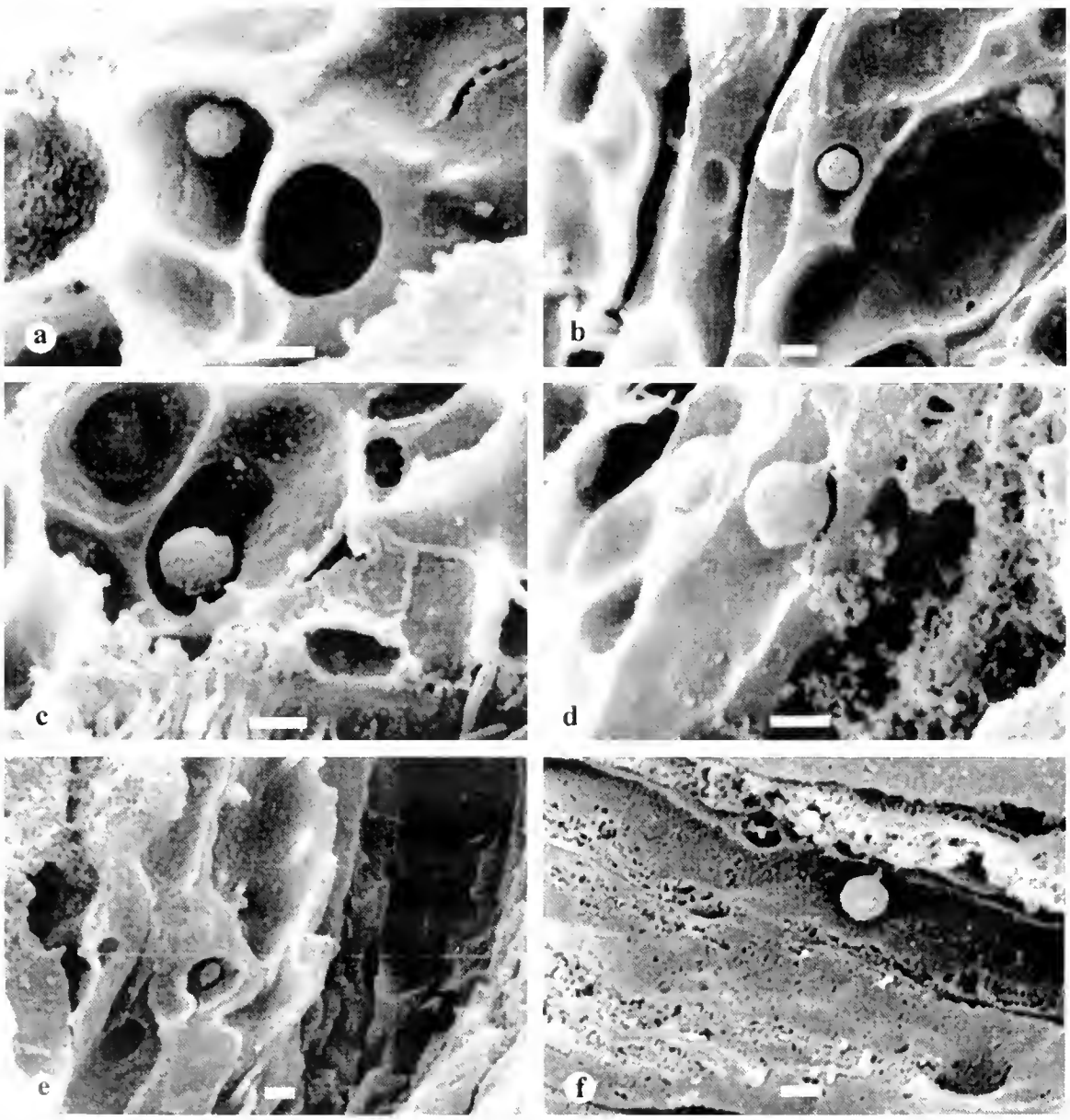

Figure 4. Scanning electron micrographs of the unidentified attached spore-like structures found in the intestinal tract of various species of freshwater unionids collected from several sites in southeast Michigan: (a) *Pyganodon grandis*, Huron River; (b) *Lampsilis siliquoidea*, Huron River; (c) *Lampsilis ventricosa*, Huron River; (d) *Ptychobranchus fasciolaris*, Huron River; (e) *Elliptio dilatata*, Vineyard Lake; and (f) Pyganodon *grandis*, Four Mile Lake. Scale bars = 1μm (a–e).

contained cellulose-, chitin-, and cellobiose-degrading bacteria, regardless of unionid species, organ from which the inoculant was obtained, or whether the sample was grown aerobically or anaerobically (Table 1). In all 336 samples, bacterial growth was noted within 12 h of inoculation based on gas production and clouding of the media. The bacterial communities developing in the cellulose and chitin media were similar in that all were motile, were fermenters, and facultative anaerobes (Fig. 5), but they differed in size. The rod-shaped bacteria growing in the chitin were longer (avg. 3.96 μm) than those in the cellulose media (avg. 2.26 μm). The bacterial community that grew in the cellobiose was dominated by non-motile cocci, which were also fermenters and facultative anaerobes. Rod-shaped bacteria were present, but even smaller in length (avg. 1.95 μm) than those found in the other types of media. However, in the December samples, none of the 112 samples inoculated in either cellulose or chitin showed any growth up to 120 hours. In contrast, the December cellobiose samples ($n=56$) did show a 100% response to this media within 12 h, regardless of unionid species, organ source, or whether the sample was grown aerobically or anaerobically.

The lake samples, *P. grandis* from Four Mile and *E. dilatata* from Vineyard, differed from Huron River unionids in that no microbial growth occurred in either cellulose or chitin media regardless of season, or organ source (total of 168 samples for both lakes) (Table 1). However, like the Huron River animals, these lake unionids consistently showed activity in 100% of the 84 cellobiose samples, regardless of season, lake, or organ source. As with the Huron River unionid cellobiose samples, the bacterial community growing on the cellobiose media was dominated by non-motile cocci that were fermenters and facultative anaerobes. In size and appearance they were identical to those observed in the Huron River unionid samples.

DISCUSSION

None of the unionids samples examined contained true endemic microflora. In order to be considered a true endosymbiont,

TABLE 1.

Growth of microorganisms removed from the lumen of mantle cavity (mantle) and digestive tract organs (gut) on cellulose, cellobiose, and chitin bacteriological media, under aerobic and anaerobic (in parenthesis) conditions.

Season	Species	Cellulose		Chitin		Cellobiose	
March	*Lampsilis ventricosa*	mantle	+(+)	mantle	+(+)	mantle	+(+)
	Huron River	gut	+(+)	gut	+(+)	gut	+(+)
and							
	Lampsilis siliquoidea	mantle	+(+)	mantle	+(+)	mantle	+(+)
	Huron River	gut	+(+)	gut	+(+)	gut	+(+)
Aug.							
	Ptychobranchus fasciolaris	mantle	+(+)	mantle	+(+)	mantle	+(+)
	Huron River	gut	+(+)	gut	+(+)	gut	+(+)
	Pyganodon grandis	mantle	+(+)	mantle	+(+)	mantle	+(+)
	Huron River	gut	+(+)	gut	+(+)	gut	+(+)
	Pyganodon grandis	mantle	0(0)	mantle	0(0)	mantle	+(+)
	Four Mile Lake	gut	0(0)	gut	0(0)	gut	+(+)
	Elliptio dilatata	mantle	0(0)	mantle	0(0)	mantle	+(+)
	Vineyard Lake	gut	0(0)	gut	0(0)	gut	+(+)
Dec.	*Lampsilis ventricosa*	mantle	0(0)	mantle	0(0)	mantle	+(+)
	Huron River	gut	0(0)	gut	0(0)	gut	+(+)
	Lampsilis siliquoidea	mantle	0(0)	mantle	0(0)	mantle	+(+)
	Huron River	gut	0(0)	gut	0(0)	gut	+(+)
	Ptychobranchus fasciolaris	mantle	0(0)	mantle	0(0)	mantle	+(+)
	Huron River	gut	0(0)	gut	0(0)	gut	+(+)
	Pyganodon grandis	mantle	0(0)	mantle	0(0)	mantle	+(+)
	Huron River	gut	0(0)	gut	0(0)	gut	+(+)
	Elliptio dilatata	mantle	0(0)	mantle	0(0)	mantle	+(+)
	Vineyard Lake	gut	0(0)	gut	0(0)	gut	+(+)
	Pyganodon grandis	mantle	0(0)	mantle	0(0)	mantle	+(+)
	Four Mile Lake	gut	0(0)	gut	0(0)	gut	+(+)

Response code: + indicates bacterial growth and media degradation occurred within 12 hours of culture; 0 indicates no bacterial growth seen from 12–96 hours after inoculation. Sample size differs and is discussed in the methods section of text.

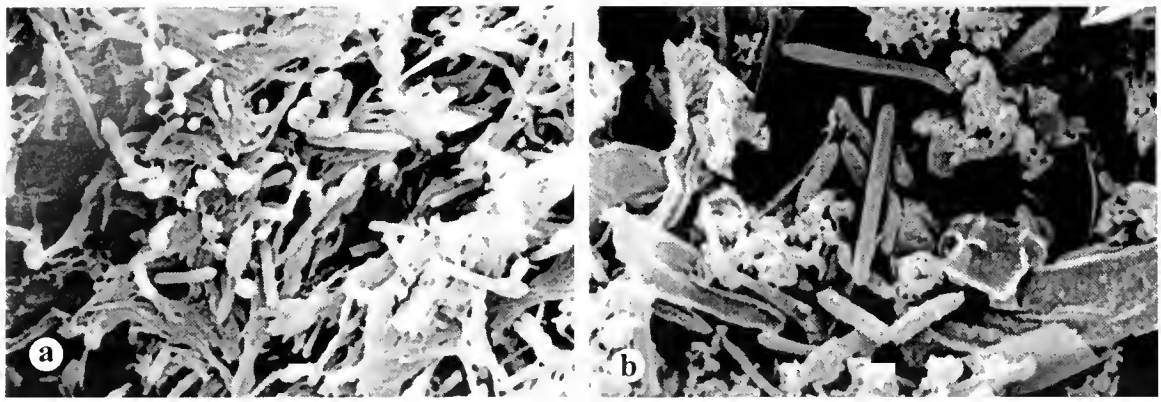

Figure 5. Scanning electron micrograph of the bacterial community cultured from the mantle cavity and gut content of freshwater Unionidae. (a) shows the bacterial community that developed in cellulose media, while (b) shows the community that developed in the chitin media. Scale bar = 1 μm.

microbial fauna must demonstrate certain characteristics which include: at least part of the endosymbiont community must be physically attached to the host's body tissues to ensure retention inside the animal; endosymbiont densities should be high ($>10^{10}$/g), and; the endosymbiont must be consistently found inside the host regardless of season or location (Yokoyama and Johnson 1993; Taylor et al. 1995). These criteria were not met. One problematic question remains regarding the unidentified spore-like structures attached or associated with every unionid intestinal tract we examined. Our inability to culture or isolate enough of these structures for further identification prevents us from labeling them as endosymbionts or to hypothesize their relationship with the unionid host. While in appearance and size these structures resemble the fruiting bodies, or zoospores, produced by fungus or a bacterium, a hyphal vegetative phase was only found once and in the mantle, not gut. The one exception, the fungal hyphae, attached to a specimen of *L. siliquoidea* (Fig. 2) and lacked spores or fruiting bodies. Even if these attached structures are spores, their limited numbers are not consistent with endosymbiont community criteria, and likely represent remnants of transient fauna. Fungi are a common component of the planktonic material drifting in the water of both Four Mile Lake and the Huron River (Nichols and Garling 2000). Occasional microbial spores are not uncommon in the intestinal tracts of some marine bivalves (Kueh and Chan 1985).

The presence of copious amounts of mucus in the mantle cavity and gut lumen of unionids (Fig. 1e) may mask or prevent the colonization of tissues by endosymbionts. Hyphal structures of fungi could easily be hidden in thick mucus layers. While surface-associated microsymbionts including fungi have been identified in many animals including fish (Mountfort and Rhodes 1991) and ruminants (Lowe et al. 1987; Sijtsma and Tan 1993) using the same techniques we used in this study, none of these vertebrates produce and utilize mucus in food handling and processing as do unionids. However, during December, when concurrent studies in the Huron River indicated unionids were not feeding (Nichols and Garling 2000), very little mucus was present inside the animals, and yet no attached microflora could be observed. A true endosymbiotic community would still have remnant, attached fauna, even when feeding was not occurring. Garland et al. (1982) have hypothesized that in oysters heavy mucus production prevents endosymbiont attachment on tissues. Such mucus hindrance might also limit the development of surface-attached endosymbiotic communities in unionids as well.

The variability of the results of attempted culture of microorganisms on cellulose or chitin media support our conclusion that unionids lack true endosymbiotic microbes capable of digesting these substrates. Microbial growth occurred on cellulose and chitin during periods that concurrent studies in the Huron River indicated the unionids were feeding (late February to late November, Nichols and Garling 2000). Similarly, microbial growth on these culture media did not occur during December when the unionids in this river were not feeding. If endosymbionts were present, we should have been able to culture them in December samples. The differences in microbial response to culture media, in combination with the fact that no microbes were found attached inside the unionids, indicate that cellulolytic and chitinolytic microbial communities represent transient fauna and not endosymbionts. The total lack of microbial growth in cellulose and chitin media by the Four Mile and Vineyard lake samples is surprising. Likely, this may correspond to significant differences in lake versus river microbial communities.

The ability to culture a microbial community on the cellobiose media from all unionids, regardless of species, season, or habitat, is certainly a trait associated with endemic microbial fauna. However, since no microbes were found attached to any unionid internal tissues, we have concluded that these starch-degrading microbes were transient fauna and not endosymbionts. While the spore-like structures found attached between the enterocytes of all unionid intestinal tracts could hypothetically be related to this cellobiose community, the lack of further identification of these structures prevents making this direct association.

Assuming primary cellulases and chitinases are not endogenously produced, the lack of cellulolytic or chitinolytic endosymbionts in unionids means that initial bond breakage must rely on transient microbial fauna. A reliance on such transient microbes could influence captive maintenance success if dry diets based on cellulose (corn/soybean) or chitin (nauplii) are fed to unionids. Efficient utilization of these feeds by these bivalves would require prior microbial predigestion in order to provide the necessary initial bond breakage to break these complex polysaccharides.

Unionids appear unique among aquatic invertebrates in that they consume detritus and yet lack microbial endosymbionts that would aid in digesting complex polysaccharides. While this may imply that detritus merely serves as a convenient substrate for preferred food items, i.e., attached epimicroorganisms, further work is needed to fully identify digestive enzymes produced by

unionids. Dependence of unionids on microbes appears complex and combines elements of selective predation, subsequent enhancement of non-prey species, and a reliance on transient fauna. Our observations do not support the endosymbiont model characteristic of well-known vertebrate/microbe associations. The role of transient microbial fauna, particularly the importance of different microbial species assemblages, may very well be key factors influencing unionid survival in relocation and aquaculture efforts.

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METHODS FOR MASS REARING STAGES I–IV LARVAE OF THE AMERICAN LOBSTER, *HOMARUS AMERICANUS* H. MILNE EDWARDS, 1837, IN STATIC SYSTEMS

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ABSTRACT We conducted a series of five laboratory experiments (7–18 days in duration) to test the interactive effects of stocking density, aeration rates, and food types on survival of American lobster (*Homarus americanus*) larvae through their first three planktonic stages (I–III) to the postlarval stage (IV). Experimental units and culture protocols were designed to replicate a 1:100 scaled-down version of equipment used in association with a fishermen-sponsored, stock enhancement lobster hatchery located in Cutler, Maine. The first four trials revealed that extremely high rates of aeration (ca. 240 mL air sec^{-1}) were necessary to distribute larvae and food sufficiently to reduce cannibalistic encounters; however, the best survival from stage I–IV (at stocking densities of 7–26 L^{-1} fed *ad libitum* with enriched *Artemia*) was only 24%. The final experiment (stocking density = 20 L^{-1}) yielded a mean survival rate (± 95% CI) of 75.8 ± 10.2% (range = 62.7% to 90.7%; n = 6). One important difference between the last and first four experiments was how stage I larvae were managed prior to their culture. In the first four trials, unfed larvae were collected from a relatively small (46 cm × 30 cm × 20 cm), screened capture basket located near the discharge pipe of a broodstock holding tank at the hatchery where they may have resided for > 12 hr. Larvae used in the final laboratory experiment were collected directly from the broodstock tank within 30 min after being liberated from the mother's swimmerets. Larvae, at relatively high densities within the screened box, likely had many more cannibalistic encounters prior to their culture than those collected directly from the broodstock tank and, therefore, suffered high rates of mortality during the first four laboratory trials. Mass rearing methods for larval American lobsters developed in conjunction with these laboratory experiments were used successfully by staff at the Cutler Marine Hatchery from 1988 to 1992. During this period, survival from stages I–IV averaged 44%, and approximately 875,000 stage IV animals were released to the wild. These culture methods have withstood the test of time as a private lobster hatchery in Maine adopted our protocols in 1993, and they continue to be in use. Further, the general techniques described here have been used since 1994 to culture European lobsters (*Homarus gammarus*) at a commercial lobster hatchery in the southeast of Ireland.

KEY WORDS: lobster, *Homarus americanus*, culture, static systems, stock enhancement, *Artemia*, microalgae, stage IV larvae

INTRODUCTION

Mass production of juvenile lobsters (*Homarus americanus* H. Milne Edwards) for stock enhancement programs began as early as 1872 in the United States (Rathbun 1886). Lobster "parks," natural tidal impoundments, were used in Maine and Massachusetts as spawner sanctuaries where egg-bearing females were held until releasing their stage I larvae. The earliest lobster hatchery was established in 1883 at Woods Hole, Massachusetts. Here, eggs were detached from females, placed into hatching jars, and stage I lobsters released to the wild (Nicosia and Lavalli 1999). In time, enhancement of lobster stocks using early planktonic individuals was abandoned in favor of methods to rear larvae for release at their earliest benthic stage (reviewed in Nicosia & Lavalli 1999).

Perhaps the most successful long-term stock enhancement effort in the United States with respect to juvenile production occurred at the Massachusetts State Lobster Hatchery and Research Station on Martha's Vineyard from 1951 to 1997. Culture protocols and equipment were developed that became the standard for production of massive numbers of stage IV individuals (Hughes 1972, Hughes et al. 1974). In particular, a 40-L round-bottom rearing vessel, or kreisel, was invented that, with sufficient flow rates (ca. 10 L min^{-1}), provided enough turbulence to distribute homogeneously both larvae and food, and minimized cannibalistic encounters (Hughes et al. 1974). Stocking these kreisels with 2,000 stage I larvae of *H. americanus* and supplying them with live brine shrimp (*Artemia salina* L.) during the ten to fourteen days in culture reportedly resulted in survival rates to stage IV between 75%–85% (Hughes et al. 1974, Serfling et al. 1974a, Schuur et al. 1976, Van Olst et al. 1980, Aiken & Waddy 1989, Waddy and Aiken 1998). Improvements on the basic design enabled the culture system to operate efficiently either using recirculating or flow-through seawater (Serfling et al. 1974a, Schuur et al. 1976, Van Olst et al. 1977). During the past three decades, the "Hughes pot," or planktokreisel, has been used widely by scientists in the United States and Canada to produce large numbers of juvenile lobsters for enhancement of wild stocks, commercial operations, and laboratory and field experiments. In addition, similar larval culture systems have been used in Europe with *H. gammarus* L. (Beard & Wickins 1992, Burton 1992, Browne 1999).

In Maine, during the mid-1980's, lobster fishers decided they wanted to fund up to five lobster hatcheries along the coast to produce stage IV individuals for stock enhancement purposes (Plante 1986). At the same time, one of us (S. Chapman) was working on a simple system to rear lobster larvae from stages I–IV in 400-L conical tanks similar to those used to culture bivalve larvae (Castagna 1983). The process involved culturing phytoplankton to enrich brine shrimp that were then added (after a period of growth) to static culture tanks containing lobster larvae. The work was carried out primarily because Chapman was unable to repeat the success of others using the smaller 40-L kreisels (Hughes et al. 1974) and he was interested in developing culture protocols that could be transferred easily to groups of fishers.

In 1986, the first fishermen-sponsored stock enhancement program for lobsters in Maine was initiated in the eastern coastal community of Cutler, and the Cutler Marine Hatchery (CMH) was created (Nicosia & Lavalli, 1999). From 1986 to 1992, this program was the only enhancement effort funded by lobster license fees through Maine's Department of Marine Resources. Although we both served as technical advisors to CMH during this time, the

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hatchery was managed by a fisherman and elderly housewife who, before that time, had no technical training in any aspect of the biological sciences.

During the first months of production at CMH in 1986, survival rates of stage I–IV lobster were very poor (< 10%). Here, we describe a series of five sequential laboratory experiments conducted between July and October 1986 that were designed to improve on this survival rate.

MATERIALS AND METHODS

A series of five larval lobster culture experiments (A–E) were conducted at ambient room temperatures at the University of Maine at Machias (UMM) between 15 July and 17 October 1986. Experiments were designed to assist staff at the CMH with culture protocols to maximize lobster larvae survival from stages I to IV. At UMM, it was not possible to replicate the culture vessels employed at Cutler's Hatchery (ca. 400-L conical-shaped fiberglass tanks filled to 375 L and aerated from the apex of the cone). Instead, we used 4.73-L plastic, flat-bottomed, round buckets (25.4 cm diameter at the top, tapering to 20.3 cm diameter at the base). A 3.2 mm hole was drilled in the middle of the bottom of each bucket (experimental unit) and a ceramic air stone fitted into each hole. A piece of tubing connected each ceramic stone to a manifold that received a supply of air from a pump (1-hp Conde, Westmoor Ltd., Sherill, NY). All experiments were conducted in 3.78 L (1 gallon) of seawater, which represented approximately 1/100th of the water volume used at Cutler. Buckets were not covered; therefore, photoperiod varied from one experiment to another. An attempt was made to determine if experimental results could be repeated at the Cutler Marine Hatchery (see Results—Experiment B).

Stage I lobster larvae used in Experiments A–D (Table 1) were collected at 0700 hr from a screened wooden box (46 cm × 30 cm × 20 cm) located near the discharge pipe of a broodstock holding tank at CMH, where they may have resided for > 12 hr. Larvae were transferred immediately to moist paper toweling nested within a stainless steel sieve (64 μ), placed inside a styrofoam cooler, and transported to UMM (a process that took between 30 to 45 min). Larvae used in Experiment E were taken directly from the broodstock tank with a small fish net only moments after they had been liberated from the mother's swimmerets (*sensu* Talbot and Helluy, 1995). Filtered (1 μ) seawater used in all experiments was collected at CMH.

At UMM, larvae were immediately transferred to a 40-L aquarium from which individuals were removed, counted, and added to experimental units containing a source of food (see below). We assumed that animals transferred to experimental units were unaffected by our handling, although we did not conduct a specific handling experiment. We used only larvae that were swimming prior to initiating each experiment. Every 48 hours until the end of an experiment, larvae in this static culture system were poured through a nylon fish net (1 mm aperture) that retained

TABLE 1.

Duration and treatments used in Experiments A (n = 4), B–D (n = 3), and E (n = 6).

Experiment	Duration[1]	Density/unit[2]	Food type[3]	Aeration rate[4]
A	15–21 July	100	LBS	Low
		100	LBS	Moderate
		150	LBS	Low
		150	LBS	Moderate
B	28 July–11 August	100	LBS	Moderate
		100	LBS	High
		150	LBS	Moderate
		150	LBS	High
		200	LBS	Moderate
		200	LBS	High
C	11–25 August	50	Clam	High
		50	FBS	High
		100	Clam	High
		100	FBS	High
		150	Clam	High
		150	FBS	High
D	4–22 September	25	Clam + LBS	High
		25	Mackerel + LBS	High
		50	Clam + LBS	High
		50	Mackerel + LBS	High
		100	Clam + LBS	High
		100	Mackerel + LBS	High
E	1–17 October	75[a]	LBS	High
		75[b]	LBS	High

[1] Experiments conducted in 1986.

[2] Number per 3.78 L (1 gallon).

[3] LBS = Live, enriched Brine Shrimp; FBS = Frozen Adult Brine Shrimp.

[4] Low = 20 mL air sec^{-1}; Moderate = 80 mL air sec^{-1}; High = 240 mL air sec^{-1}.

[a] Octagonal strips (33 cm × 33 cm) of black plastic mesh (6.4 mm aperture) added to each experimental unit

[b] Control: no plastic strips added to experimental units

larvae and any uneaten food. Larvae were returned immediately to a clean (rinsed with 60° C freshwater) bucket containing 3.78 L of seawater (room temperature) and food.

Live brine shrimp (*Artemia salina*), used as a source of food in Experiments A, B, D, and E, were reared in 40-L glass aquaria at UMM. *Artemia* were fed algae (Tahitian *Isochrysis galbana* Parke [clone T, iso] and *Chaetoceros gracilis* Ehrenberg) *ad libitum* that had been cultured at CMH. Brine shrimp were cultured for four days (total length ca. 2–3 mm) at a temperature of 30 C under constant fluorescent lighting before transferring to experimental units (ca. 40 brine shrimp per mL). In all experiments, there was always an excess number of live brine shrimp in experimental units after 48 hours. This number was estimated by counting a 1 mL sample from each unit using a Sedgewick-Rafter cell. These excess brine shrimp were returned to the experimental unit from which they had come and new, four day-old brine shrimp were added to bring the density to 40 mL^{-1}.

In Experiments A and B (Table 1), aeration rate was one of two factors tested. Altogether, three aeration rates were examined (Low = 20 mL air sec^{-1}, Moderate = 80 mL air sec^{-1}, High = 240 mL air sec^{-1} as measured crudely using a water displacement technique). Qualitatively, the low flow rate resulted in a few small air bubbles per second. Water movement was minimal and restricted to a small column the width of the air stone (ca. 20 mm) in the center of each bucket. At low aeration, both larvae and brine shrimp were visible; however, lobster larvae appeared to congregate on or near the bottom of the buckets. With moderate aeration rates, water motion also was restricted to the center of the buckets, although the diameter of the circulation zone was larger (ca. 80 mm). At this flow rate, larvae were moved in a gentle upward motion and then immediately downward in a vertical circulating current. High rates of aeration resulted in vigorous vertical and lateral movement of larvae throughout the water column. Larval motion was too rapid to observe individuals. At these aeration rates, the water surface within each bucket was moving so rapidly that it appeared to be boiling.

Experiments A–D were completely factorial with two fixed factors per trial. Experiments were sequential in that results from a particular trial were used to design the ensuing trial. In Experiments C and D, food type was varied. For Experiment C, 6–8 mm pieces of fresh soft-shell clam, *Mya arenaria* L., mantles and similar sized frozen adult brine shrimp were used to feed lobster larvae. In Experiment D, 6–8 mm pieces of fresh clam and frozen and thawed muscle tissue from mackerel (*Scomber scombrus* L.) were added to experimental units containing live, algal-enriched brine shrimp. In Experiment E, octagonal strips (33 cm × 33 cm) composed of extruded, black plastic netting (6.4 mm aperture) were positioned at a 45° angle within six experimental units. Netting extended from the bottom of the experimental unit to a few cm above the surface of the seawater. The purpose of the netting was to increase spatial heterogeneity within the experimental units and provide a surface or substrate for larvae to encounter and, potentially, reduce conspecific cannibalistic encounters. Another six units served as controls (no netting).

For Experiments A–D, a two-factor, model I analysis of variance (ANOVA) was performed on the angular-transformed percent survival data (Sokal and Rohlf 1995). Orthogonal, *a priori* contrasts were performed for each source of variation with more than a single degree-of-freedom (see Results). A single-factor, model I ANOVA was performed on the angular-transformed percent survival data from Experiment E. In all cases, the transformation served to either normalize the data (Shapiro-Wilks test [SAS 1998]) and/or homogenize variances (Cochran's Test [Winer et al. 1991]).

RESULTS

Experiment A

The experiment was terminated on day seven because excessive heat inside the laboratory on 21 July (32° C), in addition to high cannibalism rates during the first six days, resulted in the deaths of 98% of the larvae. Only 41 stage III individuals of the initial 500 larvae stocked in the experimental units (Table 1) were alive on 21 July. There was no cumulative effect on survival to this stage due to density ($P = 0.094$); however, nearly 9× as many larvae survived the seven-day trial in experimental units receiving moderate (37 animals) versus low (4 animals) rates of aeration ($P = 0.021$).

Experiment B

Seawater temperature varied throughout the experiment from 19° to 24° C, which is within the range of natural conditions experienced by *H. americanus* larvae (Van Olst et al. 1980). Salinity was constant at 31 ppt. Eight days after the experiment was initiated, a mean of at least 88% mortality had occurred in all treatments (Table 2). By this date, all surviving larvae ($n = 216$) had reached stage III in their development. On 11 August, fourteen days after the experiment was initiated, only 124 of 900 (13.8%) larvae remained across all six treatments of which 119 (96%) had metamorphosed to stage IV. ANOVA revealed that all main and interaction sources of variation were significant ($P < 0.05$; Table 3). The effect of aeration rate on mean survival depended on stocking density. *A priori* contrasts indicated that 7.7% of the larvae reared at 100 $unit^{-1}$ (ca. 26 L^{-1}) survived from stage I to IV independent of aeration rate. This was nearly twice the average

TABLE 2.

Percent survival (±95% CI) of larval lobsters in Experiment B (28 July to 11 August 1986).

Density	Aeration	Day 6	Day 8	Day 10	Day 12	Day 14
100	Moderate	21.5 (12.35)	9.7 (1.47)	7.3 (1.43)	6.0 (4.31)	5.7 (2.87)
	High	18.0 (9.43)	12.7 (6.25)	11.3 (6.22)	10.7 (6.25)	9.7 (6.25)
150	Moderate	13.3 (8.72)	5.6 (6.69)	4.9 (6.27)	2.7 (4.38)	1.9 (1.65)
	High	18.6 (6.65)	10.4 (3.40)	7.8 (7.47)	9.2 (8.29)	6.9 (4.17)
200	Moderate	15.5 (6.95)	5.5 (5.46)	4.7 (2.58)	3.9 (4.30)	3.2 (2.46)
	High	9.8 (7.56)	7.3 (10.03)	4.8 (4.70)	4.5 (1.25)	3.2 (3.99)

Density = number per 3.785 L. Aeration (Moderate = 80 mL air sec^{-1}; High = 240 mL air sec^{-1}). Day refers to days after hatching. $n = 3$.

TABLE 3.

ANOVA on the angular-transformed survival data from Experiment B (11 August).

Source of variation	df	Sum of squares	Mean square	F value	Pr > F
Aeration	1	66.59	66.59	17.43	0.0013
Density	2	106.76	53.38	13.98	0.0007
(100 vs. [150 & 200]/2)	1	100.28	100.28	26.26	0.0003
(150 vs. 200)	1	6.48	6.48	1.70	0.2172
Density × Aeration	2	36.47	18.23	4.77	0.0298
(100 vs. [150 & 200]/2) × moderate vs. high	1	0.44	0.44	0.11	0.7412
(150 vs. 200 × moderate vs. high)	1	36.03	36.03	9.44	0.0097
Error	12	45.83	3.82	—	—
Total	17	255.65	—	—	—

A priori contrasts appear with density and interaction sources of variation. See Table 2 for aeration rates. $n = 3$.

survival rate (3.8%) of larvae at the two higher densities ($P = 0.0003$). High aeration rates significantly improved survival rates of larvae stocked at 150 unit^{-1} (40 L^{-1}) compared with moderate aeration rates, but this same improvement did not occur for larvae initially stocked at 200 unit^{-1} (53 L^{-1}).

Because this experiment had shown that high aeration rates (ca. 240 mL air sec^{-1}) and a density of 100 L^{-1} may result in 5–10% survival, a two-week test of these culture variables was initiated in three 400-L conical tanks at CMH from 11 to 25 August. Culture vessels at Cutler were treated similarly to those in the laboratory. We used algal-enriched, live brine shrimp (40 mL^{-1}) as a source of food and added approximately 10,000 stage I larvae to each vessel, which was aerated vigorously. A regression equation was developed using mass (g) and larval number to estimate number of stage I larvae stocked per conical tank (Fig. 1a) and number of stage IV larvae (Fig. 1b). These equations enabled us to estimate percent survival on a per-tank basis over the two-week culture period at CMH.

Culture conditions at Cutler were very similar to those at UMM. Seawater temperature varied between 20° and 24°C and salinity was 31 ppt during the two-week interval. Survival of stage I to IV larvae in the three conical tanks was: 7.5%, 10.2%, and 4.9%.

Experiment C

Previous experiments had demonstrated that mortality between stages I and III was severe (usually > 90%) in both the large conical tanks at CMH and the experimental units at UMM. Because we observed many cannibalistic encounters during the first week, one possible explanation for this might be that larvae preferred a larger food particle than the 2–3 mm brine shrimp.

Seawater temperature varied between 21.5° and 24°C and salinity fluctuated between 31 and 34 ppt. On 15 August, four days after the experiment was initiated, survivorship summed across all treatments was 24.1% (Table 4). When the experiment was terminated on 25 August, mean survivorship over all treatments was 1.5%. ANOVA (Table 5) demonstrated both a density and food type effect. Larvae survived best (2.4%) at the lowest density (50 unit^{-1}) and frozen brine shrimp provided a significantly higher survival rate (1.9%) than chopped soft-shell clam (1%) ($P = 0.0307$, Table 5).

Experiment D

Because the extremely poor survival rates observed in the previous experiment suggested that larger food particles, alone, should be rejected as a source of food for culturing lobster larvae, we examined the interactive effects of diet (large food particles [6–8 mm pieces of clam and mackerel] together with live brine shrimp) and stocking density (25, 50, 100 larvae unit^{-1}).

Seawater temperature varied between 17° and 23°C and salinity between 31 and 35 ppt during the 18-day trial. On 16 September, an experimental unit from the low-density fish treatment was found without water. All larvae inside were dead and this replicate was removed from the experiment. During the final two days of the experiment (20 to 22 September), all four larvae found in one experimental unit from the high-density fish treatment appeared to have become infected with the ciliate *Vorticella* spp. No other larvae appeared to have the ciliate.

Unlike previous experiments, overall mortality did not exceed 80% until the 16th day of the experiment (20 September, Table 6). The experiment was terminated on day eighteen when 96% of the individuals had metamorphosed to stage IV (Table 7). Because it is not possible to separate efficiently stage III from stage IV individuals, Table 7 can be used as a guide in developing strategies for releasing early benthic phase *H. americanus* from hatcheries (such as Cutler) that do not have space to rear stage IV + animals to larger sizes either individually or communally. For example, on Day 14, 35% of the animals were at stage III while 65% were at stage IV ($0.65 \times 180 = 117$ stage IV individuals). Two days later, 94% of the remaining larvae were stage IV ($.94 \times 143 = 134$). The additional 48 hours yielded 15% more stage IV individuals even though the percent of individuals reaching the fourth stage increased over this same time interval by nearly 30% (65% to 94%). This suggests that both overall percent survival and percent of individuals that have attained the first benthic stage should influence decisions when to release larvae to the wild.

ANOVA (Table 8) was unable to detect a difference in mean survival between the two food types (clam + live brine shrimp = 19% vs. mackerel + live brine shrimp = 13%, $P = 0.084$). There was no overall effect due to density; however, the *a priori* contrast of low (22%) vs. the mean of the two higher densities (13%) was significant ($P = 0.048$).

Although mean survival at the lowest density was better than in previous experiments (Table 6), these rates were much lower than published accounts of lobster larval survival to stage IV, either by other public hatcheries (e.g., the Massachusetts State Lobster Hatchery at Oak Bluffs, Martha's Vineyard, MA, Hughes et al., 1974) or research laboratories (Serfling et al. 1974; Schuur et al. 1976; Aiken et al. 1981; Eagles et al. 1984).

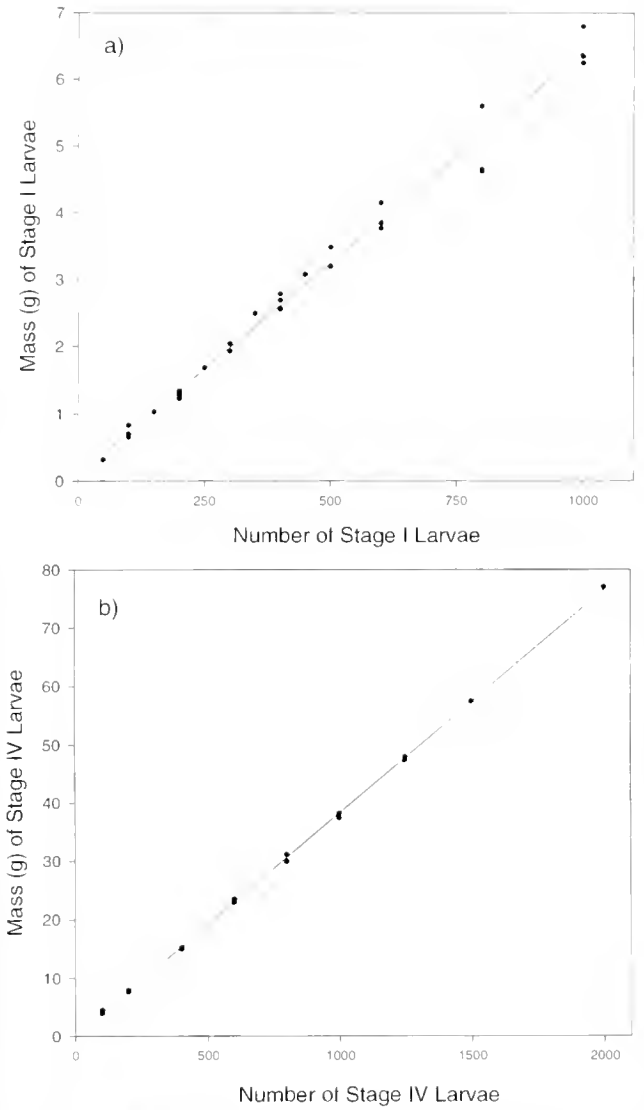

Figure 1. Mass(g)-to-number relationships for stage I (a) and IV (b) larvae of *Homarus americanus*. (Stage I: Y = 0.0824 + 0.00633 X, n = 30, r^2 = 0.988, 157.9 larvae g^{-1}; Stage IV: Y = 0.0896 + 0.03821 X, n = 16, r^2 = 0.999, 11.2 larvae g^{-1}). Larvae of both stages were caught using a nylon mesh (1 mm aperture) and transferred to a sieve (125 μ). A piece of paper toweling was used to absorb moisture from the bottom of the screening for 30 seconds before measuring mass using a Sartorius electronic balance to the nearest 0.01 g.

Experiment E

Seawater temperature varied between 17° and 22°C and salinity between 30 and 33 ppt. For no apparent reason, all larvae within one of the buckets containing a strip of the plastic netting died within the first 36 hours. On Day 15, 83% of all surviving individuals had reached stage IV. When the experiment was terminated 48 hours later, 98% had metamorphosed to stage IV. Dramatic differences in survivorship occurred between this test and all previous experiments (Fig. 2). By the end of the experiment, 18.1 ± 13.9% (95% CI) (range = 6.7% to 34.7%) of the animals in the experimental units containing plastic strips had survived to stage IV whereas 75.8 ± 10.2% (range = 62.7% to 90.7%) of the larvae attained stage IV successfully in the control units. These survival rates were highly significantly different ($P < 0.0001$)

Transfer of Techniques from the Laboratory to the Cutler Marine Hatchery

In 1986, an estimated total of 20,000 stage IV lobsters were released from CMH. The best survival rates observed during that season, which lasted from mid-June to mid-September was 12.5% (25 August to 9 September; Beal, pers. obs.). In 1987, using techniques learned from Experiment E (above), approximately 93,000 stage IV animals were released from CMH during a similar time interval. Survival rates were as high as 60%, but averaged 42% for the year (Beal, unpubl. data). From 1988 to 1992 (the last year CMH operated), an average of 175,000 stage IV lobsters per year were released by staff at CMH into coastal waters near Cutler, Maine (Beal et al. 1998). During that time, survival in the 400-L conical tanks varied between 25% and 65% with an average of 44%.

DISCUSSION

Experimental results and observations involving the initial handling of stage I lobster larvae described here led to the adoption of culture techniques that enabled staff at Cutler's Marine Hatchery to become successful in rearing *Homarus americanus* larvae through their planktonic stages (I–III) to first benthic stage (IV) for stock enhancement purposes. That program operated from 1986 through 1992 when public funding for lobster hatcheries in Maine ceased. The culture techniques described below have been in use since 1993 by staff at a private facility in Maine (the Southwest Harbor Oceanarium, SHO) who are engaged in stock enhancement efforts in the waters near Bar Harbor, Maine (T. Montague, hatchery manager, SHO, pers. com., 7 July 2000). In addition, similar culture techniques have been adopted for *Homarus gammarus* production of stage IV animals for stock enhancement efforts in Ireland (Browne & Mercer 1988).

In Maine, wild broodstock were obtained from fishers on an as-needed basis. Maine's Department of Marine Resources issued special permits annually to those who were active in the CMH stock enhancement program. (Aiken and Waddy [1985] reported that in Canada, it was difficult to obtain egg-bearing females from fishers. This led them to develop methods to domesticate broodstock for year-round seedstock production). In eastern Maine, broodstock with eggs ready to hatch (i.e., those that display a light bluish hue), were commonly available beginning the second week of June through mid-September from 1986 to 1992 (Beal, pers. obs.). Beginning in 1987 and continuing through the 1992 season, egg-bearing females entering CMH were immediately bathed in a 1% solution of Betadyne (povidone iodide) in filtered (1 μ) seawater to remove fauna and ectoparisites such as copepods, nematodes, or *Vorticella* spp., as well as reduce the probability of bacterial infections. The bath consisted of immersing the entire animal, except its mouthparts and gills, for 8–10 min. Egg-bearing females were then moved to a static, communal tank (1,320 liters) that was completely covered to provide constant darkness. No more than five female lobsters were held in this tank at any time. Ambient, filtered (1 μ) seawater in the tank was changed every two days and the tank thoroughly cleaned on a weekly basis. Hatching occurred generally throughout the day, but the hatching period for a particular female lasted no more than five days (see Hughes and Matthiessen 1962). Stage I swimming larvae were taken from the broodstock tank within 30 min of being expelled from the clutch using a small nylon aquarium fish net. Sometimes, large batches of stage I larvae were available early in the morning

TABLE 4.

Percent survival (±95% CI) of larval lobsters in Experiment C (11–25 August 1986).

Density	Diet	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
50	*M. arenaria*	30.0 (4.97)	13.3 (22.39)	5.3 (5.73)	2.0 (0.00)	2.0 (0.00)	2.0 (0.00
	Brine shrimp	30.0 (30.22)	16.7 (28.68)	8.0 (9.94)	4.7 (2.87)	3.3 (5.74)	2.7 (2.87)
100	*M. arenaria*	20.0 (7.45)	7.0 (9.93)	3.0 (4.97)	1.7 (3.79)	1.0 (2.48)	0.7 (1.43)
	Brine shrimp	30.7 (19.92)	13.3 (10.34)	8.3 (8.69)	4.3 (7.99)	2.0 (2.48)	2.0 (2.48)
150	*M. arenaria*	18.0 (7.22)	8.0 (3.32)	3.3 (2.87)	2.2 (3.45)	0.7 (1.66)	0.4 (0.96)
	Brine shrimp	15.6 (9.98)	6.4 (3.82)	2.9 (2.52)	2.2 (0.95)	1.3 (0.00)	0.9 (0.96)

Density = number per 3.78 L. Aeration (High = 240 mL air sec^{-1}). Diet (6–8 mm pieces): soft-shell clam (*M. arenaria*) or frozen brine shrimp. Day refers to days after hatching. $n = 3$.

(i.e., after CMH staff came to work ca. 0700); however, these larvae were not added to culture tanks. Instead, they were liberated because results from Experiments A–E suggested that a dense assemblage of unfed stage I larvae would not do well under culture conditions.

After collecting recently hatched larvae, each netted batch was weighed to provide an estimate of numbers placed into a 400-L culture tank (Fig. 1a). Over a two-day period, between 7,500 and 10,000 stage I larvae were added to a vigorously aerated (from the apex of the cone) conical tank filled to ca. 375 L (ca. 20–27 larvae L^{-1}). Tanks contained 275 L of seawater (18°–20°C) plus 100 L of a 1:1 mixture of the flagellate Tahitian *Isochrysis galbana* and the diatom *Chaetoceros gracilis* or *C. mulleri* Ehrenberg at a cell density of $2–4 \times 10^6$ cells mL^{-1}. In addition, lobster larvae received a supply of live, four day-old *Artemia* (initially 40–50 mL^{-1}) that were cultured and enriched at CMH.

Approximately 80 mL of dry brine shrimp eggs (Aquarium Products®, Glen Burnie, MD) were first decapsulated (Bruggerman et al. 1980) and then added to a 400-L conical tank containing only 100 L of filtered (1 μ) seawater (25°–29°C). After brine shrimp had hatched, the tank was filled with cultured algae (as above). After four days, the tank was drained and brine shrimp (ca. 3 mm) retained on a small sieve, the contents of which were added to one 400 L conical tank with larval lobsters.

Lobster larvae remained in the static culture tanks for two days before being transferred to an adjacent, clean tank containing vigorously aerated seawater, microalgae, and brine shrimp. The transfer process lasted no more than two minutes. We devised a dip net that was approximately 40 cm long × 30 cm wide × 20 cm deep made from nylon window screening. To transfer larvae, we would dip once from the larval tank and immediately empty the contents of the net into the clean tank. After six to seven dips, 95% of the larvae had been transferred. Next, we turned off the air and drained the seawater from the larval tank, quickly capturing the remaining larvae on a sieve (500 μ). This method also retained uneaten, live brine shrimp that also were added to the clean tank along with the lobster larvae. This transfer process continued for approximately two weeks, or until 85–90% of the surviving larvae had reached stage IV, when they were released to the wild. It never was necessary to add any antibiotics or other medication to a culture tank. From 1988–1992, survival rates from stage I to IV at CMH averaged 44%. These early benthic phase animals were released at depths of 10–20 m on or near cobble bottom in along a 170 km stretch of coast from Cutler (44°14′N, 67°27′W) to Tenant's Harbor (43°33′N, 69°03′W) Maine.

The larval culture techniques employed at CMH were different from methodologies that had been used to that date (Hughes et al. 1974; Serfling et al. 1974a; Schuur et al. 1976; Van Olst et al. 1980) primarily because of work conducted at the University of Maine's Darling Marine Center (DMC, Walpole, ME) from 1983-1985 by one of us (S. Chapman). Prior to the mid-1980's, most culture work on planktonic American lobsters was conducted in 40 L round-bottom, fiberglass tanks known as kreisels, planktokreisels, or "Hughes pots," after work published by John Hughes, former director of the Massachusetts State Lobster Hatchery (MSLH; Oak Bluffs, Martha's Vineyard, MA; Hughes et al. 1974) and his colleagues in California (Serfling 1974b; Ford et al. 1975; Van Olst et al. 1977). Highest estimated survival rates to stage IV per kreisel (35–85%) occurred when stage I larvae were stocked at densities between 500 and 3,000 (12.5–75 L^{-1}) and fed either live or frozen *Artemia* throughout the culture period (Hughes et al. 1974). Van Olst et al. (1980) reported that, "When food and larvae are uniformly distributed and proper densities of both are maintained, larval survival to stage IV will average 60–75%." Schuur et

TABLE 5.

ANOVA on the angular-transformed survival data from Experiment C (25 August).

Source of variation	df	Sum of squares	Mean square	F value	Pr > F
Density	2	61.28	30.64	6.58	0.0118
(50 vs. [100 & 150]/2)	1	53.02	53.02	11.39	0.0055
(100 vs.150)	1	8.26	8.26	1.77	0.2075
Food	1	27.88	27.88	5.99	0.0307
Density × food	2	6.86	3.43	0.74	0.4990
Error	12	55.87	4.66	—	—
Total	17	151.89	—	—	—

See Table 4 for description of food types. $n = 3$.

TABLE 6.

Percent survival (±95% CI) of larval lobsters in Experiment D (4–22 September 1986).

Density	Diet	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16	Day 18
25	*M. arenaria*	46.7 (20.69)	37.3 (15.18)	37.3 (15.18)	37.3 (15.18)	33.3 (11.51)	28.0 (17.21)	24.0 (9.94)	24.0 (9.94)
	S. scombrus	41.3 (50.02)	37.3 (50.02)	33.3 (59.89)	30.7 (54.73)	24.0 (57.74)	24.0 (57.74)	22.0 (49.25)	20.0 (42.75)
50	*M. arenaria*	38.0 (21.66)	31.3 (17.42)	31.3 (17.42)	28.0 (14.91)	24.0 (4.97)	24.7 (7.62)	20.0 (17.91)	20.0 (17.91)
	S. scombrus	36.0 (34.42)	25.3 (25.00)	25.3 (25.00)	23.3 (20.08)	20.0 (9.94)	17.3 (7.59)	13.3 (7.59)	12.0 (4.97)
100	*M. arenaria*	33.0 (11.40)	22.0 (13.83)	19.7 (15.97)	17.3 (21.13)	13.7 (13.68)	15.3 (21.13)	13.3 (14.98)	13.0 (14.90)
	S. scombrus	40.0 (2.48)	30.0 (7.46)	29.0 (6.57)	27.7 (6.25)	23.7 (1.44)	12.7 (11.74)	8.0 (11.38)	7.0 (12.91)

Density = number per 3.785 L. Aeration (High = 240 mL air sec^{-1}). Diet (6–8 mm pieces): soft-shell clam (*M. arenaria*) or mackeral (*Scomber scombrus*) in combination with four day-old, enriched live brine shrimp (*Artemia salina*). Day refers to days after hatching. $n = 3$.

al. (1976) reported that a maximum survival rate of 70% can be attained when 1500 larvae from a single day's hatch are added to a "Hughes pot" and are fed live brine shrimp over a two-week period at temperatures of 20.0 ± 0.5°C. Although a number of studies used these culture techniques (Hughes et al. 1974; Serfling et al. 1974a, Schuur et al. 1976), none indicated how final numbers of stage IV animals were counted, none provided estimates of mean survival or variability, and none described how larvae were handled prior to stocking each kreisel.

One of us (B. Beal) visited MSLH in November 1991. At that time, it was reported by staff that survival rates of larval lobsters from stages I to IV at MSLH typically were in the range of 15–20% and rates had been at those levels for many years (M. Syslo, director, MSLH, pers. com., 7 Nov. 1991). At that time, 40-L planktokreisels were being used as culture vessels and frozen brine shrimp used as a food source. Stocking densities of stage I larvae were estimated to be 2,000 per culture vessel.

Several published accounts from Canada suggest good success in rearing American lobsters from stage I–IV in planktokreisels (Aiken et al. 1981; Aiken and Waddy 1989; Aiken and Waddy 1995; Eagles et al. 1984; Waddy 1988; Waddy and Aiken 1998). Aiken and Waddy (1995) indicated that a diet of frozen brine shrimp will produce survival rates of 35–65%; however, Eagles et al. 1984 noted that these highly variable survival estimates were related to the quality of frozen brine shrimp. Waddy and Aiken (1998) reported that maximum stocking density of stage I larvae into 40 L kreisels at the St. Andrews Biological Station (St. Andrews, New Brunswick) was "50–75 larvae per liter" (2,000–3,000 per kreisel). Best survival rates occurred at 20°C when live adult brine shrimp were maintained at a ratio of four/lobster larvae. Although Waddy and Aiken (1998) gave no specific survival rates, they referred to Schuur et al. (1976) for a range of estimates from stage I to IV (60–75%).

According to Serfling et al. (1974a), a system to mass culture *Homarus americanus* larvae must: (1) provide water circulation enough so as to suspend both food and lobster larvae without eddies or still-water pockets; (2) use a filtration system that maintains a well-oxygenated and high water quality independent of the seawater source; (3) be operable on a fully-closed, recirculating basis for several weeks; (4) function without continuous surveillance and operate independent of outside electrical power and water supplies; (5) have temperature control; (6) have provisions for effective control of diseases; and (7) be relatively maintenance free, inexpensive, and easy to build. The system used at CMH and elsewhere (see below) was developed primarily to enable non-scientists working in non-academic or non-research settings to culture *H. americanus* larvae to stage IV. Although the culture parameters outlined by Serfling et al. (1974a) have been successful in some settings, they assume a degree of technical sophistication (especially the filtration and recirculating systems) that usually does not exist in the situations we have been working for the past two decades (i.e., with fishers and their family members).

Our culture approach uses large volumes of air to suspend and disperse both lobster larvae and food (live, enriched brine shrimp) inside large, 400-L cone-shaped tanks. These methods depend on clean seawater, and function well for two-day intervals before the entire system must be cleaned and larvae transferred to another tank. Because CMH was relatively small ($60 m^2$), we found the most efficient method of keeping seawater temperature reasonably constant was to use an air conditioner. One of the most important

TABLE 7.

Percent of larvae at each stage on each sampling date for Experiment D.

Day	Date	T°C	Percent in larval stage				Total Alive	Total % Alive
			I	II	III	IV		
1	4 Sept	20.0	100				1050	100.0
4	8 Sept	20.1		95	5		422	40.2
6	10 Sept	21.7		71	29		297	28.3
8	12 Sept	19.8		34	66		286	27.2
10	14 Sept	20.8		25	73	2	263	25.0
12	16 Sept	18.9		1	76	23	215	20.5
14	18 Sept	17.6			35	65	180	17.1
16	20 Sept	19.3			6	94	143	13.6
18	22 Sept	18.8			4	96	136	13.0

TABLE 8.

ANOVA on the angular-transformed survival data from Experiment D (22 September).

Source of variation	df	Sum of squares	Mean square	F value	Pr > F
Density	2	252.02	126.01	3.87	0.0533
(25 vs. [50 & 100]/2)	1	161.01	161.01	4.95	0.0480
(50 vs. 100)	1	91.01	91.01	2.80	0.1227
Food	1	117.46	117.46	3.61	0.0840
Density × food	2	4.19	2.09	0.06	0.9379
Error	11	358.01	32.54	—	—
Total	16	731.68	—	—	—

Refer to Table 6 for specific information about food type. $n = 3$.

pieces of information we discovered during our experimental trials that subsequently influenced our culture practices was in the timing of collection of the stage I larvae from the broodstock tank. We have been unable to find many specific references in the literature concerning this aspect of culturing lobsters. Aiken and Waddy (1995) state, "Hatching larvae are screened from the outflow of the maternal female's tank each evening and stocked in specially designed larval tanks (planktokreisels) that disperse water in an upward rotation that keeps the larvae in uniform suspension, reducing injury and cannibalism." Sastry (1975) and Chang and Conklin (1993) each describe a broodstock holding/larval collection system similar to those used at CMH in 1986. That is, filtered seawater flows unidirectionally into individual compartments holding egg-bearing females, which carries stage I lobsters into plexiglass catch baskets (no size given) with 1 mm mesh screening. "Each morning the removable catch baskets are examined for larvae, and, if present, the larvae are rinsed into a larval-rearing system" (Chang and Conklin 1993). Baskets facilitate the ease with which stage I larvae are collected and transferred to kreisels; however, we abandoned their use at CMH after 1986. We found both experimentally and in routine culture situations that, because of their cannibalistic tendencies, stage I larvae, if confined to small areas at high densities without *Artemia* or other food, either die immediately or become stressed or injured, resulting in high rates of mortality (i.e., > 50%) within the first week of culture (e.g., Experiments B–D). If capture or collection baskets were emptied often enough (i.e., before a critical density occurred within each), they would be useful. Instead, we opted to place 3–5 broodstock at a time in a small (1320 L), covered, static, lightly–aerated tank and collected larvae periodically (every 30–60 min) through the day using small, aquarium, fish nets. Stage I larvae hatched during the evening were not added to culture tanks at CMH; instead, they were liberated in a cove adjacent to the hatchery.

The culture methods outlined here are not specific to the American lobster. In 1992, a lobster (*Homarus gammarus*) stock enhancement effort was initiated in Ireland at the National University of Ireland, Galway Shellfish Research Laboratory (SRL) in Carna (53° 18′ N, 9° 49′ W). The system was based on using 40-L kreisels (Hughes et al. 1974) that were connected to a heated seawater reservoir system (18°C). Stage I larvae (density = 25–37 L^{-1}) were suspended by a continuous flow of recirculated seawater at 9 L min^{-1} and fed a combination of chopped mysid shrimp (*Neomysis* spp.), mussel (*Mytilus edulis* L.), and occasionally supplemented with live *Artemia*. After 17 days, survival rates were 9.8% (Browne 1999). In March 1993, the culture system was converted to one more closely resembling the one at CMH (Browne 1999; Beal, pers. obs.). Similar mass-to-number relationships were discovered for stage I *H. gammarus* larvae (Browne 1999) so that careful stocking estimates could be obtained. After 11–14 days in the hatchery, Browne and Mercer (1998) observed an average larval survival rate to stage IV of 47%.

The culture techniques used at SRL were transferred to a fishermen's cooperative lobster hatchery in southeast Ireland during the mid-1990's. In August 2000, one of us (B. Beal) visited this hatchery (Carne, County Wexford, Ireland) where lobsters have been produced for stock enhancement since 1994. The culture methods used are similar to those described by Browne and Mercer (1998) and Browne (1999). Stage I larvae are collected soon after release by the female and are placed into vigorously aerated seawater (18°–21°C) within 80 L conical tanks (hoppers) at densities

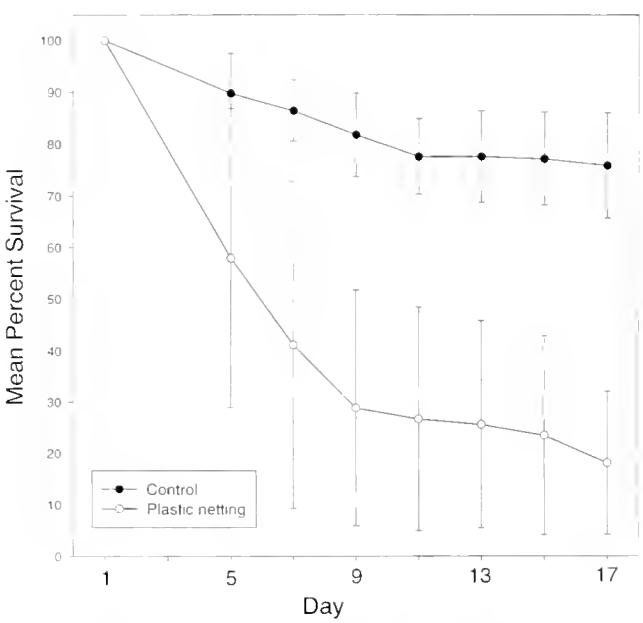

Figure 2. Experiment E. Mean percent survival ± 95% CI of *Homarus americanus* larvae through time for control (●) ($n = 6$) experimental units and those containing a piece of extruded, plastic netting (○) (6.4 mm aperture, $n = 5$). Larvae were stocked at 75 $unit^{-1}$ (ca. 20 L^{-1}), fed four day-old live brine shrimp and received vigorous aeration (i.e., 240 mL air sec^{-1}) during the entire experimental interval. Experiment was terminated on day 17 after 98% of larvae had metamorphosed to stage IV.

of 1500–2000/tank (19–25 L^{-1}). Tanks contained two species of cultured phytoplankton (Tahitian *Isochrysis galbana* and *Chaetoceros gracilis*) and live, algal-enriched brine shrimp. Larvae are transferred every second day to freshly prepared hoppers. Survival rates of lobster larvae from stage I to IV average 45% according to hatchery staff (N. Perrella, hatchery manager, pers. com., 24 August 2000).

Culture of *H. gammarus* for stock enhancement also took place in the United Kingdom in Wales and Scotland for nearly two decades beginning in 1973 (Beard et al. 1985, Lee and Wickins 1992, Wickins 1998). Larvae, stocked at 25 L^{-1} and fed twice daily with mysid shrimp (*Neomysis* spp.) and supplemented three times per week with newly hatched *Artemia* nauplii (Beard and Wickins 1992), were cultured in 100 L modified Hughes pots (planktokreisels) containing 80 L of seawater (16°–19 C). Flow rates of 7.5 to 12 L min^{-1} kept larvae and food suspended for the 11–17 days it took to reach stage IV. Survival rates (10–15%) of stage I to IV at the Ministry of Agriculture, Fisheries and Food's (MAFF) Fisheries Laboratory at Conway, North Wales were much lower than those reported from the two culture facilities in the Republic of Ireland (Beard et al. 1985, Beard and Wickins 1992). Similar survival rates were obtained at MAFF's Seafish Marine Farming Unit in Ardtoe, Scotland by Burton (1992).

The culture techniques described here are relatively simple, straightforward, and have withstood the test of time in a variety of hatch-and-release settings both in Maine (*Homarus americanus*) and Ireland (*H. gammarus*). Experiments A–D and the first year of production at CMH (1986) demonstrated that keeping lobster larvae and food well suspended within culture containers is important, but, without careful management of stage I larvae (i.e., collecting for culture within 30–60 min of release by the female), typical survival rates from stage I to IV will be less than 15%. Our techniques that produced a range of survival rates from 60% to 90% in the laboratory are not intended to replace those described elsewhere (Hughes et al. 1974, Serfling et al. 1974a, Van Olst et al. 1977, Aiken and Waddy 1989, Lee and Wickins 1992). These, too, have been used successfully in a number of disparate locations and production scenarios. Rather, we believe the culture methods described here provide another option for fishers, mariculturists, or research scientists to produce large numbers of stage IV clawed lobsters for stock enhancement, commercial enterprise, or experimental purposes.

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EFFECTS OF DIET ON *NEPHROPS NORVEGICUS* (L.) LARVAL AND POSTLARVAL DEVELOPMENT, GROWTH, AND ELEMENTAL COMPOSITION

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ABSTRACT Few studies have been conducted on the culture of *Nephrops norvegicus* larvae and none of them have permitted significant numbers of postlarvae to be obtained (stage IV). The goal of this study was to determine a suitable diet for rearing *N. norvegicus* to juvenile stage V. Survival, molting periodicity, growth, and elemental composition (CHN) of the young postembryonic stages were used as criteria of physiological condition. Four different diets, fresh enriched nauplii of *Artemia* (FENA), artificial feed for shrimp (AF), commercial frozen mussels (M), and commercial frozen adult *Artemia* (FAA) were tested from hatching to stage V at ambient temperature (14–16 C). Five day old stage I larvae fed with AF, M or FAA showed a similar biomass as freshly hatched larvae, indicating that the energy derived from food only allowed them to survive but not to gain length. FENA was the only diet that resulted in development to stage V. Larvae fed with FENA presented the shortest time of development, the highest rate of survival, and the highest growth rates in size and biomass.

KEY WORDS: *Nephrops*, spiny lobster, larva, growth, diet, C,H,N

INTRODUCTION

The commercial fisheries of the Norway lobster, *Nephrops norvegicus* (Linnaeus, 1758), are economically important in Europe, and many projects regarding this species have been developed with the support of the European Community (ICES 1999, Sardà 1996). In the Mediterranean Sea, the production and average size of *N. norvegicus* have decreased during the past 10 years, while fisheries effort has increased (Franquesa 1996, Lleonart 1995, Sardà 1998a). Growth, reproduction, molt and feeding have recently been studied in the Mediterranean Sea (Sardà 1998b). Despite the economical importance of this species, information concerning the larval stages is scarce compared to that for adults and to other commercial lobster as the species of the genus *Homarus* Weber, 1795. The early postembryonic development consists of three larval stages (I, II, III). These were morphologically described by Sars (1884, 1890) from Norwegian waters, by Jorgensen (1925) from the northeast coast of England, and by Santucci (1926a,b,c, 1927) from the Tyrrhenian Sea. The larval phase is separated by a metamorphic molt from the postlarva (stage IV), which presents the morphological characters of the adult. The postlarvae swim for a few days before becoming benthic. There is still a controversy about the terminology of lobster development; some authors suggest that stage IV is already a juvenile. Others prefer to call stage IV a postlarva or decapodid and stage V the first juvenile (see Phillips & Sastry 1980, Charmantier et al. 1984).

Although larval distribution has been studied in different regions of the Atlantic Ocean and the Mediterranean Sea from a fisheries point of view (Brown et al. 1995, Eiriksson 1970, Farmer 1975, Lindley 1983, Milligan & Nichols 1988, Nichols et al. 1983, Nichols et al. 1987, Thomson et al. 1986), only a few studies have been conducted on requirements for the development of *N. norvegicus* larvae and postlarvae. Figueiredo and Vilela (1972) and Figueiredo (1971) reared *N. norvegicus* larvae in the laboratory at different salinities (33–40‰) and temperatures (7–17°C). In this study, the larvae seemed to be unaffected by salinity in the tested range, while increasing temperature accelerated not only growth, but mortality as well. Larvae reared at 11–14°C presented the highest survival (25% to stage II, 13% to stage III and 7% to stage IV) and juveniles lived as long as three months reaching 18 mm in total length. Increased temperatures also reduced the intermolt period, 14–15 days at 7–10°C to 5–6 days at 13–17°C. *Artemia* Leach, 1819 nauplii proved to be insufficient as a diet and better growth was achieved with eggs of *Crangon crangon* (Linnaeus, 1758) as food. Thompson and Ayers (1989) tested temperatures ranging from 5°C to 15°C finding the same effects as Figueiredo and Vilela (1972) and Figueiredo (1971). The best results were observed at 15°C, where survival was 12% from stage I to stage II, 40% between II and III, and 8% between III and IV. In addition, the estimated duration of the intermolt period was 6.9 days from stage I to stage II, 6.1 days between II and III, and 9.0 days between III and IV. In these studies, small *Artemia* sp. were provided as a food, occasionally complemented with wild plankton and encapsulated feed. Recently, Dickey-Collas et al. (2000), reared *N. norvegicus* larvae at different temperatures from 8.6°C to 14.7°C with a mixed diet of *Tetraselmis suecica* and freshly hatched *Artemia* nauplii. Mortalities were greater than 50% by the end of stage I at all temperatures tested and only one larvae was raised to stage IV at 11.2°C. Stage I lasted from 7 to 18 days and no correlation with the rearing temperature could be made. Survival exceeding 90% to stage III was achieved by feeding the larvae with larger *Artemia* juveniles (Anger & Püschel 1986), but this rearing experiment was not continued thereafter. Development time was 8.7 days for stage I, 11.3 days for stage II, and 11.5 days for stage III. The elemental composition (CHN) of the larvae did not change very much during development. Only freshly hatched larvae showed significantly lower percentages of organic constituents than older larvae.

Hence, further information about the culture conditions of *N. norvegicus* is critical to successful commercial culture. Aquacul-

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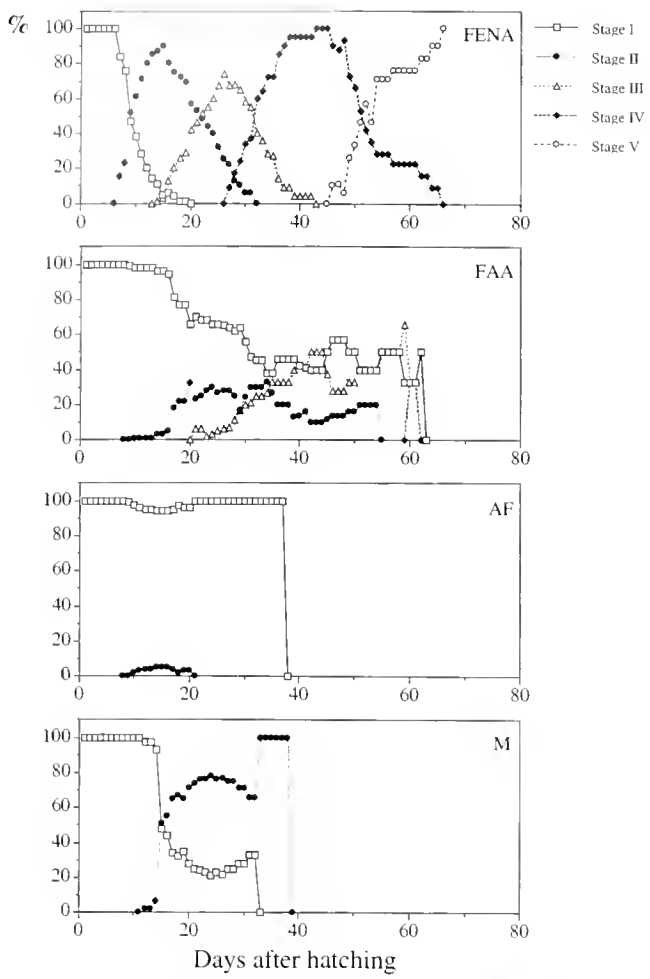

Figure 1. Percent survival and occurrence of successive stages over time for larval and postlarval development of *Nephrops norvegicus* reared with four different diets: fresh enriched nauplii of *Artemia* (FENA— after hatching, nauplii were enriched for 24 h with lipids and vitamins using DHA Selco, INVE); artificial feed for shrimp, DIBAQ (AF); commercial frozen mussels (M); and commercial frozen adult *Artemia* (FAA).

ture of nephropid lobsters has been reviewed by Van Olst et al. (1980), showing that efforts to develop techniques for the culture in controlled environmental systems have been concentrated on species of the genus *Homarus*. By comparison with *N. norvegicus*, a large body of information is now available on the culture and physiology of *Homarus* (review in Factor 1995).

Generally, poor nutritional quality has been shown to increase the time of development and to decrease survival and growth of lobster larvae (Conklin 1995). The goal of the present study was to improve growth, molting, and nutrition of *N. norvegicus* larvae and postlarvae from hatching to stage V, and to evaluate the prospects of a culture to complement the capture fishery.

MATERIALS AND METHODS

Culture of Larvae and Postlarvae

Ovigerous female Norway lobsters were caught off Barcelona harbor by a commercial fishing fleet at 400 m depth from January to March. They were brought to the laboratory and maintained for 1 to 3 weeks in individual cylinder-conic containers protected by a mesh to avoid escape of freshly hatched larvae. These containers were placed in a 3000 L tank with running sea water at a temperature of 15 ± 1°C and 12 h light/12 h dark photoperiod. Females were fed with frozen mussels twice a week.

After hatching, 2,169 larvae from a group of 30 females were cultured individually in connected boxes fed with running seawater at ambient temperature (15 ± 1°C). Four different diets were tested:

1. FENA: fresh enriched nauplii of *Artemia* AF 480 (21.3 mg/g DW HUFA & 0.04 DHA/EPA). After hatching, the nauplii were enriched for 24 h with lipids and vitamins using 300 mg/L of DHA Selco (200 mg/g DW HUFA & 2.96 DHA/EPA) made by INVE AQUACULTURE NV (Oeverstraat 7, 9200 Baasrode, Belgium).
2. FAA: commercial frozen adult *Artemia* produced by Mundo Acuático, S.L. (C/ Llobregós 9, 08032 Barcelona, Spain).
3. AF: artificial feed for shrimp (feed granules of 0.2 mm containing 12% lipids, 15% carbohydrates, 52% proteins, vitamins, oligo-elements, and 5.500 Kcal) produced by DIBAQ AQUACULTURE (Polígono Industrial "Baix Ebre" Parcela 114, Camp-redó, 43897 Tortosa, Spain).
4. M: commercial frozen mussels.

Food was provided *ab libitum* twice a day. The different treatments were applied homogeneously through the circuit boxes in order to ensure that differential mortality was not influenced by the rearing system.

Growth Rate and Molting Frequency

Survival and molting of larvae and postlarvae in each treatment were recorded daily. Carapace length (CL) was measured to the nearest 0.1 mm directly from photographs or with a dissecting microscope for larvae fixed in 4% formol. Carapace length of postlarvae was measured with callipers. Estimates of growth parameters were based on the model of Von Bertalanffy (1938), as this curve has been commonly accepted for adults (e.g. Mytilineou and Sardà 1995, Sardà 1985). For fitting the curve, FISHPARM software was used (Prager et al. 1987). The slopes and intercepts of the different growth curves were compared by applying an ANOVA.

Measurements of Biomass

Larvae from 6 different females were taken immediately after hatching and on day 5 (stage I, intermolt stage C) after feeding with the test diets (FENA, FAA, M, AF). Biomass was measured as wet weight (WW), dry weight (DW), carbon (C), nitrogen (N), and hydrogen (H). The CHN values are presented as absolute (per individual) and relative contents (CHN in % of DW), and as C/N and C/H ratios.

Samples were briefly rinsed in distilled water, blotted on fluff-free filter paper, and WW was measured to the nearest 0.01 mg on an Ohaus AP250D–0 balance. Thereafter, the material was stored at −80°C. Before the analysis, the samples were freeze-dried overnight in a GT 2 (Leybold-Heraeus) apparatus and weighed again to the nearest 0.1 μg on a Mettler UM 3 balance to obtain DW. Larvae were combusted at 1020°C in a CHN analyser (Carlo Erba Science, Model 1108, Version Fisons).

The arithmetic means and the standards errors of biomass were calculated from 4 to 8 replicates. The significance of differences

between mean values of freshly hatched and 5 day old larvae fed different diets were determined using a one-way analysis of variance (ANOVA) followed by a multiple test at a probability level of $p<0.05$.

RESULTS

Molting Frequency

Among the four diets tested, only the FENA diet permitted development to stage V. Commercial frozen adult *artemia* allowed development to stage IV, while AF- and M-fed larvae reached only stage II (Fig. 1). In addition, only larvae fed with FENA showed a regular pattern of molting and development through the successive stages. Commercial frozen adult *Artemia* fed larvae showed a higher rate of survival until day 30 as compared to the other diets. Thereafter, however, the larvae fed with FENA had a slightly higher survival.

Survival and duration of the developmental stages are given in Table 1. Survival rate from stage I to stage II was 21.3% with FENA, 0.9% with AF, 9.4% with M, and 5.4% with FAA. The average time of development from hatching to stage II was 9, 10, 16, and 18 days, respectively, with FENA, AF, M, and FAA. With the two *Artemia* diets, some larvae reached stage IV; the time to reach stages III and IV was 20 and 32 days with FENA, and 30 and 60 days with FAA, respectively.

Growth Rate (Increments in Body Size)

Larvae fed with AF and M survived for up to 40 days, with high variability in length (Fig. 1) and in larval size (carapace length, CL). Larvae and postlarvae fed with FENA or FAA, in contrast, survived for over 70 days (Fig. 1). The Von Bertalanffy growth curves (VBGC) could be fitted only in these two groups (Fig. 2). Their slopes were significantly different ($P<0.001$), showing that the larvae fed with FENA showed a higher rate of growth than those fed with FAA.

Changes in Biomass

Biomass changes (WW, DW, CHN per individual) measured between freshly hatched and 5 day old larvae fed with different diets are documented in Table 2. Changes in the water content (% WW) and in the relative elemental composition (CHN in % of DW; C/N and C/H ratios) are documented in Table 3. Weight and elemental composition (C, N) are shown in Figure 3 for freshly hatched (fh) and 5 day old stage I larvae of one female (no. 23), where sufficient material from all dietary conditions was available.

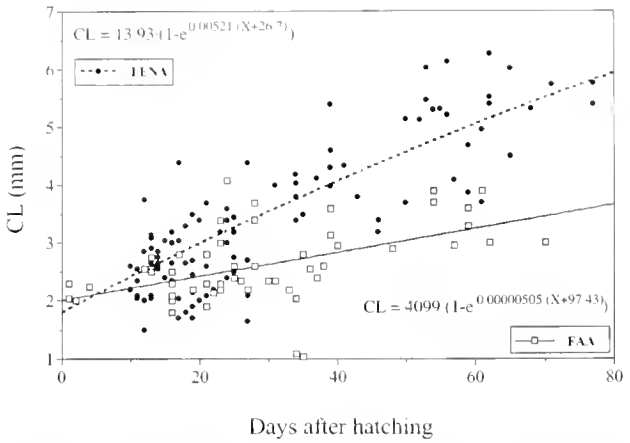

Figure 2. Comparision of Von Berlantanffy growth curves (VBGC) for *Nephrops norvegicus* larvae and postlarvae reared under two different nutritional conditions: fresh enriched nauplii of *Artemia* (FENA) and commercial frozen adult *Artemia* (FAA). See Figure 1 for further details.

This comparison shows that all measures of biomass per individual (WW, DW, C, N; H is not included but shows the same trends) were consistently highest in the FENA group. WW reflected the dietary quality less clearly. Likewise, the percentage CHN figures (in % of DW) were significantly higher in the FENA treatment than in all others. The water content was on average lower in larvae fed with FENA than in the other treatments. In 5 day old FAA-fed larvae, all biomass values were consistently below those of freshly hatched larvae; these losses were statistically significant in the percentage of C, N, and H values (Table 3, Fig. 3).

Hence, FENA was the only diet that allowed the larvae to reach a significantly higher biomass within 5 days of hatching. With FAA, M, and AF, the larvae conserved the initial biomass they already had at hatching but did not gain weight. No significant differences in the C/N and C/H ratios were observed between freshly hatched larvae and those fed for 5 days with any of the test diets. In addition to nutritional effects, significant differences were detected between larvae originating from different females (see Tables 2 and 3).

DISCUSSION

The rate of survival to the juvenile stage V was very low (maximum 0.6%) with all diets. Figueiredo and Vilela (1972) and

TABLE 1.

Survival and duration of the larval and first postlarval stages of *Nephrops norvegicus* fed with different diets.

Diet	*n*	Survival Stage I (%)	Duration Stage I (days)	Survival Stage II (%)	Duration Stage II (days)	Survival Stage III (%)	Duration Stage III (days)	Survival Stage IV (%)	Duration Stage IV (days)
FENA	521	21.30	8.81	27.93	10.92	41.93	12.23	53.85	19.71
FAA	859	5.47	17.71	31.91	12.09	10.00	6.67	0	–
M	352	9.37	15.73	0	–	–	–	–	–
AF	437	0.92	10.5*	0	–	–	–	–	–

FENA: Fresh enriched nauplii of *Artemia* (after hatching, nauplii were enriched for 24 h with lipids and vitamins), FAA: commercial frozen adult *Artemia*, M: commercial frozen mussels, and AF: artificial feed for shrimp.

* Only two molts observed.

TABLE 2.

Changes in wet weight (WW), dry weight (DW), carbon (C), nitrogen (N) and hydrogen (H) in *Nephrops norvegicus* larvae freshly hatched or fed with different diets.

			WW (mg)		DW (mg)		C (mg)		N (mg)		H (mg)	
Female	Condition	*n*	$\bar{x}$	±SD	$\bar{x}$	±SD	$\bar{x}$	±SD	$\bar{x}$	±SD	$\bar{x}$	±SD
5	Freshly hatched	4	5.66	0.39	0.656	0.095	0.207	0.035	0.046	0.007	0.031	0.006
23	Freshly hatched	5	5.35	0.39	0.508	0.102	0.158	0.031	0.035	0.007	0.023	0.005
23	FENA	8	5.69	0.56	1.001	0.157	0.349	0.063	0.079	0.015	0.056	0.011
	FAA	5	5.80	0.43	0.699	0.117	0.220	0.053	0.050	0.011	0.034	0.009
	M	5	5.31	0.33	0.667	0.092	0.192	0.032	0.045	0.009	0.028	0.005
	AF	5	5.12	0.29	0.538	0.075	0.150	0.020	0.034	0.006	0.025	0.006
21	FENA	2	5.63	0.04	0.831	0.123	0.261	0.042	0.061	0.007	0.040	0.007
	FAA	5	5.85	0.22	0.536	0.090	0.157	0.032	0.036	0.007	0.023	0.005
	M	5	4.65	0.95	0.586	0.059	0.166	0.020	0.040	0.005	0.025	0.003
	AF	5	4.70	0.78	0.564	0.087	0.160	0.021	0.038	0.005	0.023	0.003
26	FENA	5	5.59	0.94	0.628	0.182	0.219	0.070	0.051	0.016	0.033	0.012
29	M	5	6.36	1.18	0.598	0.101	0.173	0.033	0.041	0.008	0.026	0.005
9	FAA	8	5.76	0.64	0.551	0.111	0.167	0.034	0.039	0.008	0.024	0.005

FENA: fresh enriched nauplii of *Artemia;* FAA: commercial frozen adult *Artemia;* M: commercial frozen mussel; AF: artificial feed for shrimp.

Thompson and Ayers (1989) found similar rates of survival with a mixed diet (nauplii and adult *Artemia*, wild plankton, or eggs of *Crangon crangon*). However, Anger and Püschel (1986) obtained a survival of over 90% to stage III with living adult *Artemia*, though they did not monitor survival after this stage (considering stage IV as the first juvenile instar). In the present study, FAA-fed larvae showed a high rate of survival through the first 30 days, but the developmental period in stage I was nearly twice that found with other diets in this study or reported by other authors. Also in another clawed lobster, *Homarus americanus* (H. Milne Edwards, 1837), the nutritional value of frozen adult *Artemia* was found to be less than living adult *Artemia* (Conklin 1995). The nutritional value of adult *Artemia* apparently decreases greatly during storage in frozen condition. Figueiredo and Vilela (1972) and Dickey-Collas et al. (2000) showed that early *Artemia* nauplii were also insufficient for *N. norvegicus* larvae, although they were successfully used as a diet for the larvae of palinurid lobsters (Van Olst et al. 1980). The intermolt duration of larvae in stage I was shorter for that fed with FENA. The duration of larva stages fed with FENA was very close to the duration observed by the other authors at similar temperatures (Anger and Püschel 1986, Figueiredo and Vilela 1972, Thompson and Ayers, 1989).

Growth has been studied in *N. norvegicus* adults and juveniles, both in natural populations and in captivity (Belchier et al. 1994, Mytilineou & Sardà 1995, Sardà 1985, Tuck et al. 1997, Verdoit et al. 1999). In the present study, the generally applied Von Bertalanffy (1938) growth curve fit for larvae and postlarvae fed with either FAA or FENA but not for the other treatments. The growth curve obtained with FENA presented a significantly steeper slope, with a CL_∞ value close to 14 mm in the fourth larval stage. Figueiredo and Thomas (1967) found that one year old *N. norvegicus* individuals reach 13–15 mm carapace length, correspond-

TABLE 3.

Changes in the percentage of wet weight (% WW), dry weight (% DW), carbon (% C), nitrogen (% N), and hydrogen (% H), and C/N and C/H ratios in *Nephrops norvegicus* larvae freshly hatched or fed with different diets.

			Water (% WW)		C (% DW)		N (% DW)		H (% DW)		C/N		C/H	
Female	Condition	*n*	$\bar{x}$	±SD	$\bar{x}$	±SD	$\bar{x}$	±SD	$\bar{x}$	±SD	$\bar{x}$	±SD	$\bar{x}$	±SD
5	Freshly hatched	4	88.38	1.63	31.58	1.14	6.97	0.32	4.74	0.24	4.54	0.19	6.66	0.13
23	Freshly hatched	5	90.39	2.37	31.15	1.23	6.99	0.32	4.58	0.24	4.46	0.04	6.81	0.09
23	FENA	8	82.15	3.79	34.75	1.41	7.83	0.4	5.54	0.34	4.44	0.14	5.61	0.15
	FAA	5	87.99	1.34	31.29	2.23	7.07	0.5	4.78	0.44	4.43	0.11	6.56	0.14
	M	5	87.46	1.23	28.7	1.49	6.68	0.5	4.26	0.27	4.3	0.12	6.75	0.11
	AF	5	89.52	1.06	27.9	1.05	6.39	0.36	4.68	1.21	4.37	0.16	6.24	1.33
21	FENA	2	85.24	2.08	31.35	0.35	7.37	0.26	4.79	0.11	4.26	0.20	6.55	0.08
	FAA	5	90.43	1.37	29.20	1.22	6.72	0.50	4.20	0.23	4.36	0.20	6.95	0.54
	M	5	86.87	3.50	28.23	0.94	6.80	0.29	4.22	0.19	4.15	0.05	6.69	0.10
	AF	5	87.80	2.39	28.53	1.05	6.81	0.40	4.15	0.12	4.19	0.09	6.87	0.11
26	FENA	5	87.63	3.18	31.75	1.58	7.38	0.39	4.80	0.38	4.30	0.08	6.62	0.19
29	M	5	90.21	2.80	28.85	1.45	6.81	0.42	4.26	0.26	4.24	0.05	6.78	0.09
9	FAA	8	90.39	2.00	30.35	1.14	7.04	0.19	4.39	0.18	4.31	0.12	6.91	0.19

FENA: fresh enriched nauplii of *Artemia;* FAA: commercial frozen adult *Artemia;* M: commercial frozen mussel; AF: artificial feed for shrimp.

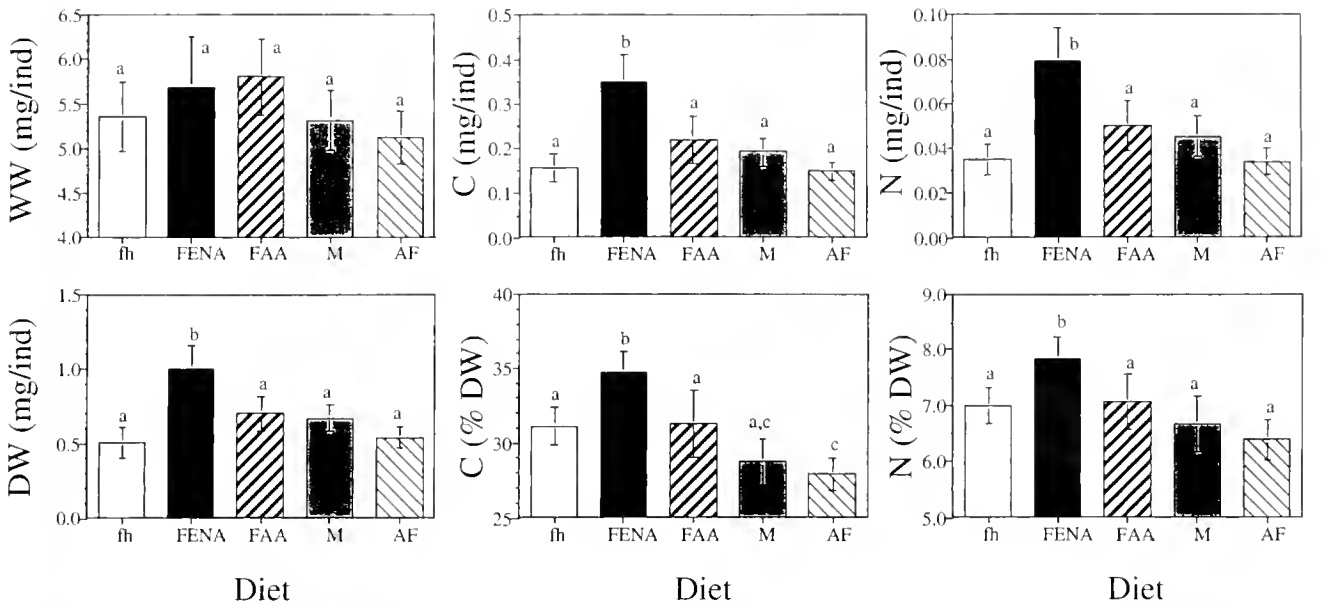

Figure 3. Body composition of freshly hatched (fh) and 5 day old *Nephrops norvegicus* stage-I larvae, reared in the laboratory under 4 different nutritional conditions: fresh enriched nauplii of *Artemia* (FENA); artificial feed for shrimp (AF); commercial frozen mussels (M); and commercial frozen adult *Artemia* (FAA). See Figure 1 for further details. Wet weight (WW), dry weight (DW), Carbon (C), and nitrogen (N) content per individual; C, N also in % of DW.

ing to 20–25 mm total length. Comparing our growth curves for larvae with those obtained by Sardà (1985) for juveniles, we can conclude that the average growth rate of larvae and postlarvae is approximately 4 to 5 times faster than in juveniles.

Feeding in homarid lobsters begins immediately after hatching and the larvae may not recover from nutritional stress if inadequate food is provided during the first stage (Anger et al. 1985, Aiken & Waddy 1995). In the present study, after 5 days of feeding with AF, M, or FAA, stage I *N. norvegicus* larvae showed similar absolute and relative CHN values and a similar water content as freshly hatched larvae. In FENA-fed larvae, by contrast, the CHN contents (both per individual and as proportions of DW) were higher and the water content (in %WW) was lower than at hatching. The CHN values measured by Anger and Püschel (1986) in successfully developing larvae of *N. norvegicus* also increased significantly after hatching. These data indicate that the energy taken from inadequate diets (AF, M, FAA) allowed only for survival but not for gaining length.

In addition, carnivorous larval decapods have limited enzymatic capabilities during the planktonic development. Since a gastric mill is absent, the survival of lobster larvae with the proteolytic enzyme levels must depend upon the high energy, easily-digestible nature of the zooplankton prey, together with an extended gut residence time. In *N. norvegicus* larvae trypsin-like activity and content per individual increases from stage I to stage III (Kumulu and Jones 1997). The CHN results obtained in the present study and the enzymatic works confirm the importance of providing high-quality food immediately after hatching to assure a successful development.

In our data, variation in the C/N and C/H ratios did not reflect the developmental stage or differential nutritional quality of the tested diets. This agrees with observations by Anger and Püschel (1986), who found that the absolute biomass increased significantly during the development of *N. norvegicus* larvae, but the relative composition (CHN in % of ash-free DW) remained fairly constant. Hence, for larvae of this species, the relative composition of CHN seems to be less sensitive as an indicator of the nutritional state than the water content.

N. norvegicus is economically a very important species and its culture is far from being accomplished, particularly when compared with other homarid lobsters. The data presented demonstrates that larvae fed with FENA showed the best nutritional condition. However, future studies in culture systems, water quality, broodstocks, and feeding are necessary to improve Norway lobster larval development.

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WIND PATTERN MAY EXPLAIN THE SOUTHERN LIMIT OF DISTRIBUTION OF A SOUTHWESTERN ATLANTIC FIDDLER CRAB

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ABSTRACT The SW Atlantic fiddler crab *Uca uruguayensis* (Nobili 1901) is distributed from the northern limit of the Argentinean Biogeographic Province (31°S to 42°S) to the northern part of Argentina (38°34′S). This distribution range seem puzzling given that this biogeographic province goes several hundred kilometers S and there are no obvious environmental discontinuities that might explain this pattern. To explore possible causes of this distribution, hypotheses related to biological and oceanographic constraints were investigated. A comparative study between the three southern sites (Bahía Samborombón, 35°30′–36°22′S; Mar Chiquita coastal lagoon, 37°46′S and Quequén Grande river, 38°34′S) where this species inhabits showed that biological constraints are not the limiting factor. There were no differences in morphometry, reproductive timing and output, body condition and larval sizes. Differences were only found on the size frequency distributions, which indicate differences in recruitment regularity. However, wind patterns relative to coastal bearings indicate that during the period of larval release and recruitment (December to April), the contribution of wind drift transport are less frequently coastward from the La Plata river (35°–36°20′S, where Samborombón is located) to the south. If the wind stress is the main force moving the upper layer of the water column and consequently crab larvae, then this pattern would indicate that there is a decrease in the chances of carrying megalopae to the coast south of the La Plata river. On the basis of this information we suggest that the southern distribution limits of this species is mainly due to wind pattern.

KEY WORDS: fiddler crabs, *Uca uruguayensis*, distribution, recruitment, wind pattern, larval supply

INTRODUCTION

The Argentinean Biogeographic Province is an homogeneous area with regards to pelagic and offshore inhabiting species (see Balech 1964, Bernasconi 1964, Stuardo 1964, Menni and Gosztonyi 1982, Boschi 2000). The southwestern Atlantic waters in this area are transitional between colder subantarctic and warmer subtropical Brazilian waters (Longhurst 1998).

The southwestern Atlantic fiddler crab *Uca uruguayensis* (Nobili 1901) is found from southern Brazil (33°S) to the northern coast of Argentina (38°S; Boschi 1964) in estuarine environments, overpassing the La Plata river (35°–36°20′S) but not reaching the southern limit of the biogeographic province. One of the southernmost permanent populations inhabits Bahía Samborombón (35°30′–36°22′S) where it is the dominant intertidal species (Boschi 1964) reaching densities of up to 140 crabs m^{-2} (Iribarne and Martinez 1999). Smaller populations are found a few hundred kilometers south, at the Mar Chiquita coastal lagoon (37°46′S, 57°27′W; Spivak et al. 1991), at the Quequén Grande estuary (38°34′S; Boschi et al. 1992) and at the Quequén Salado estuary (38°56′S; pers. obs.; Fig. 1). This distribution pattern seems puzzling, given the lack of obvious environmental features (e.g., large rivers or obvious changes in current pattern) that may explain it. However, even when the causes are unknown the processes that determine this pattern should be either biological limitations (e.g., McConaugha 1992) or physical factors affecting larval distribution (e.g., Roughgarden et al. 1988). The main purpose of this work is to explore these alternative hypotheses.

Several authors have discussed the existence of limitations in the species distribution (see Hengeveld 1992), particularly in species with dispersion through larvae. Geographic distribution can be limited by biological factors (e.g., Menge 1991), hydrographic processes that modify the supply of larvae (e.g., Farrell et al. 1991), relationship between these two options (e.g., Connolly & Roughgarden 1998) or environmental factors such as air temperature, tidal range, rainfall (Crane 1975) and sediment characteristic (Frith & Brunenmeister 1980).

Some *Uca* species produce planktonic larvae that are exported to open ocean and then return to recolonize their habitats (Lambert and Epifanio 1982, Epifanio 1988). Success of recruitment depends on larval survival in the planktonic phase and subsequent settlement in the adult habitat (McConaugha 1992), which may largely depend on nearshore hydrodynamic processes (Underwood & Denley 1984, Farrell et al. 1991). On the basis of a comparative analysis between the three southernmost populations as well as wind field analysis on several years, as the most possible factor responsible of coastal circulation, the purpose of this work is to investigate this general hypothesis. Specifically, we state and explore two sets of hypothesis that may explain the southern distribution limit of this species: (a) biological limitation and (b) oceanographic constraints.

MATERIALS AND METHODS

Study Sites

The study was conducted in three sites (Fig. 1): (1) the mouth of the easternmost tidal channel (San Clemente tidal inlet;

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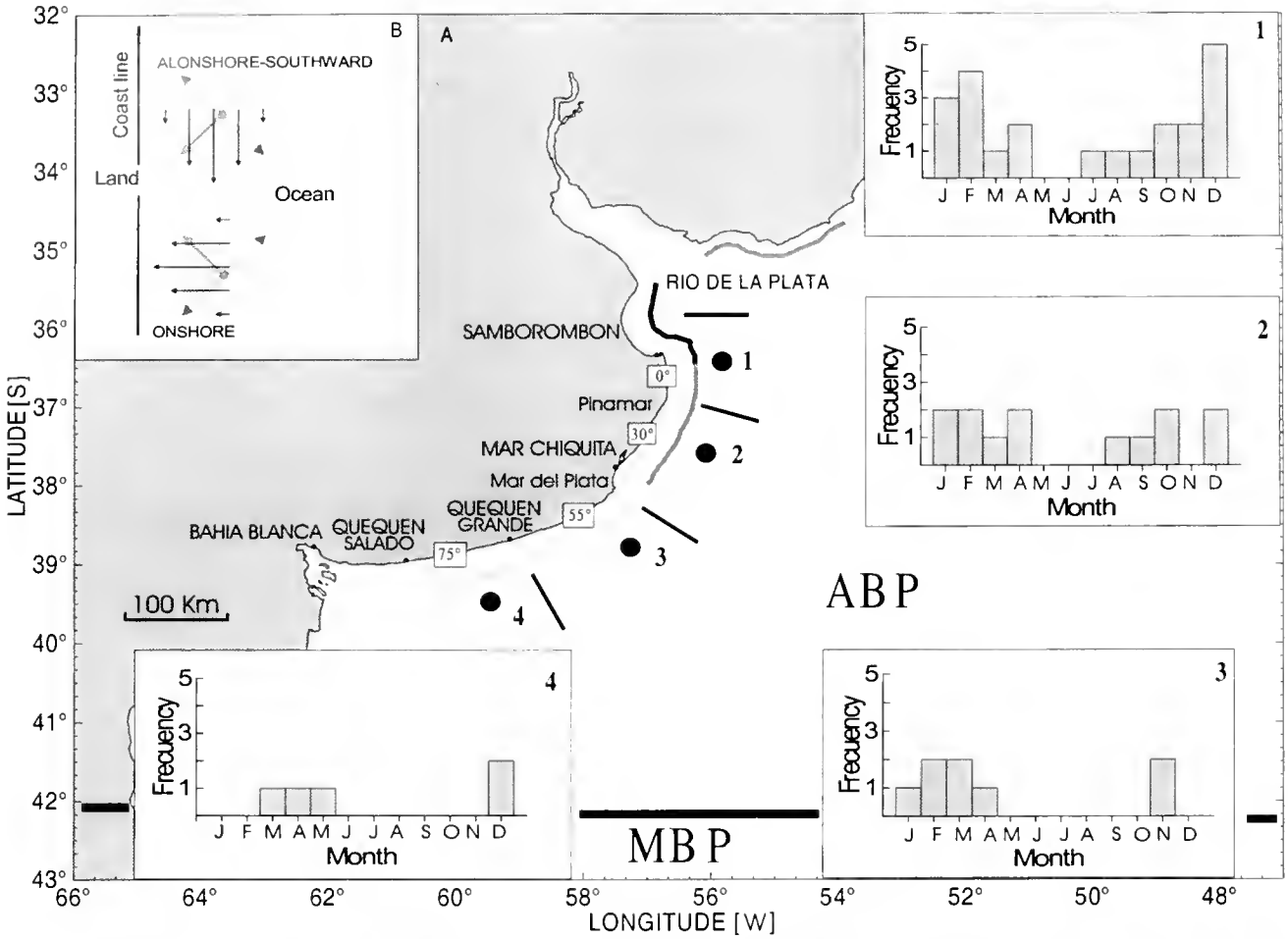

Figure 1. (A) Geographic position of the study sites: Samborombón bay, Mar Chiquita coastal lagoon, Quequén Grande river, Quequén Salado river. Line along the coast represents geographic distribution of *Uca uruguayensis*. ABP: Argentinean Biogeographic Province, MBP: Magellanic Biogeographic Province. Grid points position for the MWF data analyzed (1 to 4). Solid lines delimit the four areas according to coastal bearing. (B) Ekman surface layer field current (dark arrows) forced by wind field (background arrows) that gives favorable drift (southward and onshore) contributing to the megalopae recruitment. Histograms 1 to 4 correspond to monthly occurrence (absolute number of records) of southward and onshore conditions during the time period analyzed.

36°22′S, 56°45′W) of the Bahía Samborombón (100 km long coastal basin within La Plata river estuary, Argentina), (2) the Mar Chiquita coastal lagoon (37°46′S; 300 km south), and (3) Quequén Grande estuary of the river (38°34′S; 200 km further south). All areas are characterized by fiddler crabs (*Uca uruguayensis*) and burrowing crabs (*Chasmagnathus granulata*) populations (Spivak et al. 1991, Iribarne and Martinez 1999). The littoral zone extends into an extensive *Spartina*-dominated saltmarsh (Bortolus and Iribarne 1999) and the fiddler crab population inhabits the upper open intertidal zone near the *Spartina densiflora* fringe, with few individuals living at vegetated areas. Information was also obtained from Quequén Salado river (38°56′S.) a site located 150 Km west of the Quequén Grande river.

Biological Characteristics

Crab samples were collected monthly from October 1998 to February 1999, covering the reproductive season (see Spivak et al. 1991). Each time, a random sample of 100 individuals was obtained to evaluate size frequency distribution, sex ratio and reproductive stage. Given that distribution of this species is patchy, to estimate density from each study site we obtained 50 sampling units of 1 m^2, 10 m apart, covering 500 m along the coast. Within dense patches (clearly noticeable crab beds) also 10 samples were obtained using the same procedure. Both sampling schemes were intended to have a measure of the mean overall density (density along the whole beach) and mean density of the *Uca* beds. ANOVA tests were used to evaluate differences between sites for both sampling strategies (Zar 1999).

Given logistic constraints, it was impossible to keep track of settlement and recruitment across the southern boundary. Therefore, for each study site, size frequency distribution as well as diverse population and individual characteristics were evaluated to consider possible post-recruitment process that could produce the latitudinal abundance failure.

From each sample, number of males and, ovigerous and non-ovigerous females were counted. All individuals were measured (maximum carapace width, precision 0.02 mm). Comparing crab body conditions from different sites, male and female (non-ovigerous and ovigerous without eggs) dry weight were considered covering homogeneously size classes found at each site. Crabs

were dried at 70 C during 48 hours, and weighed (precision 0.001 g). The null hypothesis of no difference in the relationship between dry weight and crab size, among categories (i.e., males, non-ovigerous females and ovigerous females) and sites, was evaluated with an ANCOVA test (Neter et al. 1990).

To compare the amount of energy allocated to reproduction between sites, carbon proportion (as ash free dry weight, AFDW) between the egg masses and the females body (without eggs) was estimated. This proportion was called "energetic allocation". AFDW was obtained by incinerating the samples at 500°C for 4 hours, after having them drying for 48 hours at 70°C. We first evaluated the relationship between "energetic allocation" and female size; given the lack of relationship we just tested the null hypothesis of no difference in the mean values using Kruskall-Wallis test (Zar 1999).

To compare egg production between sites, eggs number were estimated from dry weight of the egg mass (precision 0.0001 g) weighing 500 eggs from each site. Two egg stages were defined: "early", when embryo were undifferentiated and the eggs were completely filled with vitellum, and "late" when embryo is differentiated, with eyes and the egg was less than 40% filled with vitellum. The null hypothesis of no difference in the relationship between carapace width and number of eggs among sites was evaluated with an ANCOVA test (Neter et al. 1990). To evaluate differences between number of early and late stage eggs for each site, the average egg mortality during development was estimated. The null hypothesis of no difference was evaluated with an ANCOVA test (Neter et al. 1990).

Early stage eggs from at least 50 females (regardless of carapace width) from each site were sampled, measuring their maximum length with an ocular micrometer (precision 0.01 mm). The null hypothesis of no difference among sites was evaluated with a Kolmogorov-Smirnov test (Conover 1980).

Morphometric characteristics were evaluated in order to examine possible phenotypic plasticity. Different body measures were also obtained from males and females from each site (50 each), in order to perform a whole morphometric comparison. From each individual we measured: (1) carapace length (CL), (2) maximum carapace width (CW), (3) minimum carapace width (MCW), (4) distance between eyes (DE) (5) merus length of the third pereiopod of the left side of females and the hypertrophied side of males (Lp1), (6) width of the fourth segment of the abdomen (AW), and (7) corporal height (CH). For males, we also obtained several measures of the hypertrophied cheliped: (8) merus length (ML), (9) carpus length (CLC), (10) total propodus length (PL), (k) propodus width (PW), (11) propodus height (PH), and (12) dactylus length (DL). Individuals of similar sizes were selected from each site to avoid the effect of size. Data were log transformed to comply with statistical analysis assumptions (Hair et al. 1995).

Sizes and morphometry of larvae were also analyzed. Five females from two extreme sampling sites (Samborombón and Quequén) were maintained in aquarium with seawater salinity of 34 at continuous aeration (following Rieger 1996). When eggs hatched, 50 larvae (zoea I) were preserved in 3% formalin. Then, the following measures were obtained: (1) total rostral length (RL), (2) frontal spine length (FSL), (3) dorsal spine total length of the (DSL) and (4) carapace length (CL). All measures were obtained with a precision of 0.02 mm. Then a discriminant analysis (DFA) (Hair et al. 1995) of both larvae and adults, was performed independently to evaluate the null hypothesis of no differences between sites.

Wind Pattern

Wind-driven coastal circulation estimated as Ekman (1905) transport was assumed to be responsible for larval transport in coastal areas (McConnaughey et al. 1992). Ocean surface monthly mean winds (Mean Wind Field, MWF) from ERS-1, ERS-2 and ADEOS-1 scatterometers database (IFREMER 1998) were employed in this analysis to infer Ekman transport. The period covered was from August 1991-February 1998, totaling 78 observations (lacking February 1992). Monthly winds were statistical interpolated using kriging minimal variance method and integrated into objective analysis (Bentamy et al. 1996) to obtain the monthly mean. Each grid points used on this analysis had an average of 8.6 satellite observations (SD = 3.6). Grid spatial resolution was 1° × 1° in latitude and longitude (111 km × 92.5 km average for study region). Accuracy reported for ERS scatterometer wind data was analyzed by comparison with *in-situ* measurements from the National Buoy Data Center, Tropical Atmosphere Ocean data and Japan Meteorological Agency buoy data (Graber et al. 1996). The root square mean error was 1.2 m s^{-1} in intensity and 24° in direction (Graber et al. 1996). NSCAT / ERS-2 correlation shown error differences about 1.1 m s^{-1} and up to 28° (IFREMER 1998).

Direction of the Ekman transport (Ekman 1905, cited by Neumann & Pierson 1966) forced by these winds was estimated at selected grid points of study area (Fig. 1A) to evaluate favorable larval drift. For the southern hemisphere the direction of pure drift current at the surface will result 45° to the left of wind vector, while the vertical integrated transport over Ekman layer will be 90° to left of the wind direction. These angles will start decreasing when water depth diminishes below 40 m where the frictional layer reaches the bottom. For this study, we considered that the transport of the upper surface layer will result 45° to the left of the wind direction taking into account the water depth off the coast ranges mostly between 30 to 50 m. This assumption was also coherent with observations on *Cancer magister* zoeae and megalopae drift off Northeastern Pacific (Lough 1976, Reilly 1983) and was applied by McConnaughey et al. (1992) on *C. magister* recruitment variability studies.

Winds were assembled by the direction of the resulting surface flow into four groups of 180° angle range, where each would force the upper water layer to have, relative to coastline, an onshore or offshore and southward or northward component. For a 0° coastline oriented N-S, an onshore (offshore) condition will result when wind blows from 45° to 225° (225° to 45°), with maximum effect with SE winds or 135° (NW or 315°; Fig. 1B). Southward (northward) flow will occur under winds blowing from the angle range 315° to 135° (135° to 315°), with an optimum displacement with NE winds or 45° (SW or 225°). Four coastal regions were associated to each grid point, where wind directions were broken down relative to a defined coastal bearing (Fig. 1A): (1) Samborombón-Pinamar (37°07′S, 57°10′W) 0° angle; (2) Pinamar-Mar del Plata 30° angle; (3) Mar del Plata-Quequén Grande 55° angle and (4) west of Quequén Grande 75° angle. Favorable *Uca uruguayensis* settlement was considered when winds force upper surface waters to move onshore and in a southward direction. For each region the wind directions that favor this drift condition were plotted throughout the year (insert 1 to 4 on Fig. 1). To a pool of absolute number of events recorded from December to April, the null hypothesis of no difference between regions in the proportion favorable:non favorable events was evaluated with multiple comparisons for proportions (Zar 1999).

Results

Biological Characteristics

Maximum density showed differences between Samborombón and two other sites (ANOVA: F = 61.98, df = 1, 33; *a posteriori* Tukey analysis $P < 0.01$). The mean density showed a latitudinal gradient, being higher at Samborombón, intermediate at Mar Chiquita and lower at both Quequén river sites (Kruskall-Wallis: H = 51.075, df = 3, 263, $P < 0.01$), no differences between Quequén Grande and Quequén Salado were found (Fig. 2).

Size frequency distribution differences between sexes were found at Quequén (Fig. 3) where males were larger. Those males were also larger than males at the two other sampled sites (Samborombón: x = 10.52 mm, SD = 1.82; Mar Chiquita: x = 10.72 mm, SD = 1.82; Quequén: x = 12.15 mm, SD = 0.89). Females of Samborombón were smaller than those from the other two sites (Samborombón: x = 9.79 mm, SD = 1.38; Mar Chiquita: x = 11.09 mm, SD = 1.22; Quequén: x = 10.95 mm, SD = 1.13).

Male proportion was higher at all sites (Samborombón: 41.7f: 58.3m ($P < 0.01$), Mar Chiquita: 36.3f:63.7m ($P < 0.05$), Quequén: 34.8f:65.2m ($P < 0.01$)), but there were no differences between sites (F = 2.14, df = 2, 526, $P > 0.05$). Although some differences were found, ovigerous female percentages showed a similar pattern through the summer at all sites (Fig. 4).

At all sites, differences in dry weight and carapace width relation between sexes were found, being higher in males and smaller in ovigerous females (Samborombón: ANCOVA: F = 105.2, df = 2, 145, $P < 0.01$; Mar Chiquita: ANCOVA: F = 31.7, df = 2, 72, $P < 0.01$; Quequén: ANCOVA: F = 80.3, df = 2, 132, $P < 0.01$; Tukey *a posteriori*, always $P < 0.01$; Fig. 5). Regarding slope, only differences were found between sites for males, being lower at Samborombón (Regression Analysis for log transformed data: Samborombón: F = 85.3, df = 47, b = 0.09, $P < 0.01$; Mar Chiquita: F = 448.1, df = 30, b = 0.14, $P < 0.01$; Quequén: F = 160.7, df = 31, b = 0.14, $P < 0.01$). High heteroscedasticity for males at Mar Chiquita and Quequén did not allow comparison. Females showed no difference between sites in slope. ANCOVA analysis showed differences in ovigerous females between sites (non-ovigerous females: F = 2.18, df = 1, 127, $P > 0.05$; ovigerous females: F = 11.15, df = 1, 118, $P < 0.01$) which were higher at Quequén (Tukey *a posteriori* test: Samborombón-Mar Chiquita: $P > 0.05$, Samborombón-Quequén: $P < 0.01$, Mar Chiquita-Quequén: $P < 0.01$).

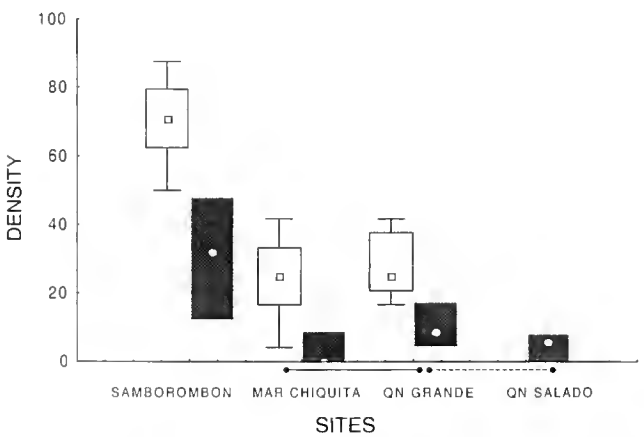

Figure 2. Density expressed as crabs m^{-2} within dense patches (MAXIMUM) and along 500 m of the coast (MEAN). Box plots are constructed with limits of boxes being the 75th and 25th percentile; lines represent 10th and 90th percentiles. Points inside boxes are medians. Boxes underlined are not significantly different between sites (ANOVA-Tukey test, $P > 0.05$; To MEAN: Kruskall-Wallis test, $P > 0.05$). Solid box: maximum density and dashed box: mean density.

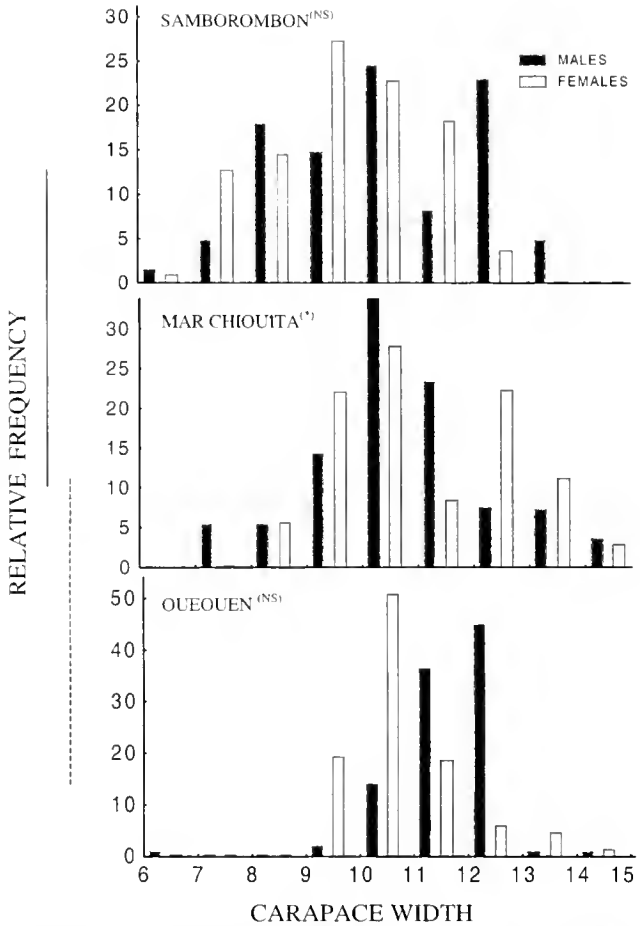

Figure 3. Size frequency distributions (carapace width, mm) of males and females, for each site. Relative frequency is the percentage of crabs of the total sample. Vertical lines parallel to axis indicate no significant difference between sites (Kolmogorov- Smirnov test, $P > 0.05$). Results of Kolmogorov-Smirnov test between sexes are indicated by: (*) $P < 0.01$ and (NS): $P > 0.05$.

There was no relationship between carapace width and energetic allocation at any site (Samborombón: t = −1.32, df = 60, $r^2 = 0.029$, pe = 1.31, $P > 0.05$; Mar Chiquita: t = 0.55, df = 23, $r^2 = 0.014$, pe = 0.88, $P > 0.05$; Quequén: t = -0.45, df = 60, $r^2 = 0.003$, pe = 1.06, $P > 0.05$). There were no differences between sites in AFDW (Kruskal-Wallis: $\chi^2 = 3.8$, df = 2, 141, $P > 0.05$).

There were no differences between sites in the relationship between female carapace width and egg number (ANCOVA: F = 1.9, df = 2, 143, $P > 0.05$ Fig. 6). Egg size was not correlated with female size at any site. The early egg size frequency distribution showed smaller sizes in Mar Chiquita (Kolmogorov-Smirnov test: Samborombón-Mar Chiquita: $P < 0.01$, Samborombón-Quequén: $P > 0.05$, Mar Chiquita-Quequén: $P < 0.01$). The relationship between female carapace width and egg numbers did not show significant egg mortality at both sites (between egg stages, Samborombón: ANCOVA: F = 0.098, df = 1, 46, $P > 0.05$; Quequén: ANCOVA: F = 0.58, df = 1, 57, $P > 0.05$).

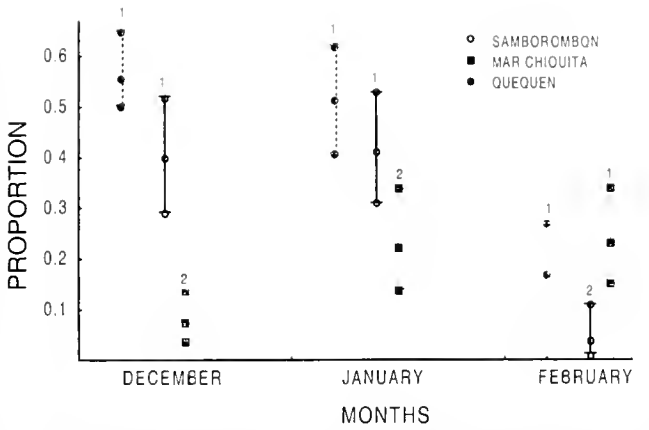

Figure 4. Proportion of ovigerous to non-ovigerous females (Ho/H) along reproductive period (December 1998 to February 1999), for each site. Relative frequency is the percentage of crabs with respect to total sample. Different numbers indicate differences between sites at $P < 0.05$ (Non-parametric multiple comparison test, NS: $P > 0.05$; Zar 1999). Segments indicates confidence limits for the proportion of ovigerous females:non-ovigerous females, for each site and month (Zar 1999).

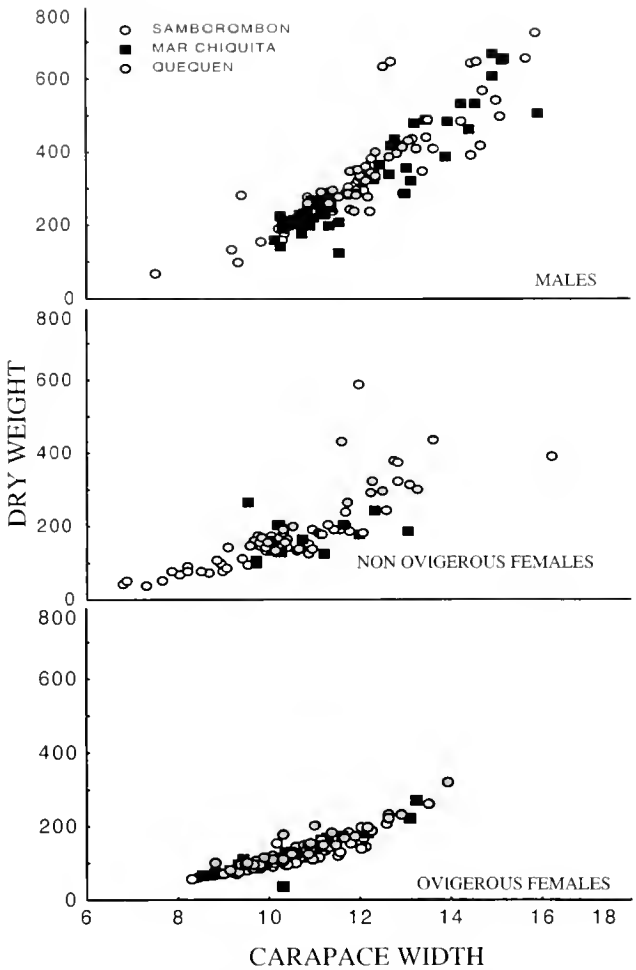

Figure 5. Relationships between size (carapace width, mm) and dry weight (mg) of males, non-ovigerous females and ovigerous females, for each site.

The discriminant analysis was unable to discriminate between different groups of *Uca uruguayensis* regardless of their geographical origin (DFA, log transformed: Males: Lambda Wilk = 0.36, F = 1.65, df = 22, 54, $P > 0.05$; Females: Lambda Wilk = 0.75, F = 1.48, df = 12, 114, $P > 0.05$). All morphometric characters had some contribution to variance, being DL and PH and CW and MCW the variable with the higher weight to males and females, respectively. Larvae discriminant analysis did not either show significant differences between sites (DFA: Lambda Wilk = 0.83, F = 2.14, df = 4, 41, $P > 0.05$). However, some differences may occur given that individuals were classified 85.2% at Samborombón while only 42.1% those from Quequén.

Wind Patterns

On the basis of satellite imagery, wind patterns that drive surface water southward and onshore were observed in the late spring and summer periods for four regions. However, these favorable conditions did not occur every year, and inter-annual variability is important. From the 46 months analyzed (from December to April) 15, 9, 6 and 4 months were favorable at 1, 2, 3, and 4 regions, respectively. There was no significant difference in proportion (favorable:non favorable events) between regions 2 and 3, and 3 and 4 (Multiple comparison for proportions: region 2:region 3, $P > 0.05$; region 3:region 4, $P > 0.05$; insert 1 to 4 on Fig. 1). Characteristic mean wind module with favorable directions for regions 1 and 2 was 6.3 m s^{-1} (SD = 0.8 m s^{-1}).

DISCUSSION

Our results show a clear pattern of decreasing density and abundance of *Uca uruguayensis* with latitude over a land distance of less than 300 kilometers. However, there are no evidences of biological differences. Reproduction shows a similar pattern, fecundity is not different, eggs and larval sizes as well as the amount of energy allocated to eggs are similar. Only a difference in large adult size frequency distribution is found.

Differences in size frequency distribution may be due to recruitment or survival, genetic differences (Hartnoll et al. 1993), food availability (Genoni 1985) and/or quality differences or food competition. Higher density at Samborombón may increase food competition and affect growth rate. Indeed lower body condition (as reflected in dry weight pattern) shown may be the result of higher density. Males and ovigerous females are individuals that require high energetic reserves (Klaassen & Ens 1993). However, better body condition found on females at the southern limit of distribution suggests that the decrease in abundance is not due to physiological constraints. Furthermore, morphometric results did not show geographic differences, suggesting that environmental condition (including diet, habitat etc.) would not be differencing phenotypic expression between sites (see Overton et al. 1997).

Sediment characteristics, organic content or humidity are recognized as important factors in determining *Uca* species distribution (e.g., Teal 1958). However the comparison of the burrowing crab effect on sediment characteristics, between Mar Chiquita and Samborombón, reveal that there are no differences in the sediment properties (Botto and Iribarne 2000), and suggests that these factors are not the basis for the density differences between sites.

Predation by shorebirds has only been observed at Samborombón (Iribarne and Martinez 1999), although other sites such as Mar Chiquita are well studied (Botto et al. 1998; Botto et al. 2000). There are four migratory shorebirds (*Pluvialis squatarola, P. dominica, Numenius phaeopus* and *Arenaria interpres*) and a tern

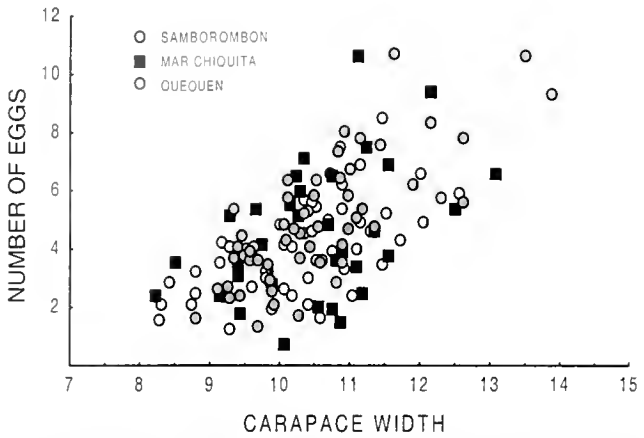

Figure 6. Relationships between number of eggs (in thousands) and carapace width (mm) of ovigerous females. For each site data of egg stage (early, late) and month (December, January, February) were pooled.

(*Gelochelydon nilotica*) that heavily prey on fiddler crabs at Bahía Samborombón, but not at the other sites (Iribarne and Martinez 1999). Although this strong predation pressure may explain small sizes at Samborombón, it does not explain the southern limit of the distribution.

Size frequency distribution at Mar Chiquita and Quequén show absence of individual corresponding to early autumn recruitment (from 5 to 7 mm, see Spivak et al. 1991). All information previously discussed does not explain such difference with Samborombón nor the latitudinal gradient density. An alternative explanation can be based on low temperature effect on post-recruitment mortality. This hypothesis is based on: (1) the fact that species of *Uca* are known to be tropical (Crane 1975) and (2) its temperature range tolerance is low (see Dezi et al. 1993, Vernberg and Tashian 1959). Although temperature can limit *Uca* species latitudinal distribution, limitations of land crab distributions by low temperature does not need to involve mass mortality since cold can limit energy available for growth (Wolcott 1988). Pattern of annual temperature suggests that during winter months the higher latitude sites seem to have a relatively lower minimum (4.7°C at Quequén and 4.8°C at Bahía Blanca, 38°43′S; Anonymous 1985, 1992); however, differences appear to be very small to support such hypothesis (maximum difference 2.7°C, between Samborombón and Quequén).

An alternative hypothesis, but not independent, is the lack of supply of larvae to the south. Larvae fate is unknown in this species, though in most *Uca* species they are exported to sea and will reinvade estuary sometime later (Lambert and Epifanio 1982; Epifanio 1988). Generally, larval movement can be entirely at mercy of currents (Butman 1987). As a consequence, larvae of coastal organisms can be transported by nearshore currents hundred of kilometers (Bertness 1999). The return of postlarvae (i.e., megalopae) to the estuary may be the problem. Settlement and recruitment process are recognized as important in determining the distribution and abundance of species ("supply side ecology": i.e., Lewin 1986) especially for benthic marine invertebrates where new individuals are supplied through settlement of pelagic larvae (Gaines and Roughgarden 1985). In this case, population size may be limited by the arrival rate of larvae. Such "recruitment limited" populations would fluctuate in size as a response to temporal fluctuations in larval settlement rate. As a result, recruitment limited populations could be defined empirically as those with significant temporal correlation between settlement rates and local population sizes (Gaines and Lafferty 1995).

In metapopulational context, larvae local populations contribute to a common larval pool, from where they are subsequently distributed among local populations (Roughgarden and Iwasa 1986). Larval transport among local populations is a function of the physics of ocean circulation, larvae behavior and duration of planktonic stages (see Gaines and Lafferty 1995). Particularly, wind forced currents have been shown to play an important role in larval transport shoreward for settlement at the appropriate time (e.g., acorn barnacles *Semibalanus balanoides:* Hawkins and Hartnoll 1982; bryozoan *Membranipora membranacea*: Yoshioka 1982; blue crab *Callinectes sapidus*: Johnson and Hester 1989). Thus, if recruitment of megalopae to the estuary depends on transport forced by nearshore wind patterns, the supply of adults would also be affected by variations in the yearly wind pattern during the critical time when larvae are offshore (Johnson and Hester 1989). Our work evaluated the year round pattern of winds driving a southward and onshore Ekman drift. During late spring and summer the larvae may reside offshore in the surface water and are affected mainly by Ekman transport due to wind stress. This phenomenon is observed for other species (e.g., American lobster *Homarus americanus*: Campbell 1989; *Callinectes sapidus:* Johnson and Hester 1989; Dungeness crab *Cancer magister:* McConnaughey et al. 1992). Wind transport influences *Uca* spp megalopae on larger open bodies of water, whereas tidal events become more important regarding the entry to the estuary (Mense et al. 1995).

Our evidence based on wind patterns suggests that supply of larvae is the limiting factor, although the coupling of the coastal ocean and marine atmosphere (exchanging buoyancy and momentum) occurs within a time scale of a week (synoptic signal). With 1-month resolution, a general trend is still observed. Synoptic signal inclusion in wind monthly mean is confirmed through the comparison between twelve months from 1993 database used, and simulated monthly mean linearly interpolated from synoptic European Center for Medium-Range Weather Forecasts analysis (ECMWF) shows that both data set are correlated between latitude 60°S to 60°N. Correlation coefficient value changes with latitude, but for the study region it is between 0.4 and 0.8 ($P < 0.05$; IFREMER 1998).

Our results show that wind condition in the coastal zone (southward and onshore) provides more favorable conditions to settlement at the northern sector of the study area than at the southern estuaries. This pattern agrees with a latitudinal decrease in *Uca* densities from Samborombón to Quequén. Recruitment (as inferred from the size frequency distribution) also is continuous at Samborombón (Iribarne and Martinez 1999), while it is not evident at southern estuaries. Ancillary information on recruitment between 1993 and 1996 at Mar Chiquita coastal lagoon (Luppi pers. obs.) shows that settlement success agrees with coastward wind pattern inferred from monthly estimates. These data show that recruitment success was high only in January 1996 (at least 39 megalopae m^{-2}), intermediate in March 1993 (21 megalopae m^{-2}), and low in January 1995 (2 megalopae m^{-2}) and March 1994 (8 megalopae m^{-2}). Furthermore, during the summer 1997 megalopae were found sporadically (Valero et al. 1999). This information coincides with favorable (1996) and unfavorable wind pattern conditions (for other years). Thus, abundance pattern is consistent with latitudinal gradient in the larvae arrival hypothesis, rather than unfavorable environmental conditions for the biology of this species. Furthermore, on the basis of results it is likely that Sam-

borombón acts as the parental stock while the other are 'satellite' populations (Hanski 1982).

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EFFECT OF BROODSTOCK DIET ON REPRODUCTIVE PERFORMANCE OF THE PEPPERMINT SHRIMP, *LYSMATA WURDEMANNI*

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ABSTRACT Four types of frozen adult *Artemia* biomass [regular and enriched (with HUFAS and other nutritional supplements) San Francisco Bay and Canadian brands], and frozen hard clam (*Mercenaria mercenaria*) were evaluated as broodstock diet for the marine ornamental shrimp *Lysmata wurdemanni*. There is no significant difference among the five diet treatments in fecundity (number of eggs produced), relative fecundity (number of eggs/g female), egg dry weight, egg percent ash, and total length of newly hatched larvae. Egg volume was significantly larger in the San Francisco Bay regular treatment than those of other treatments. Survivorship from zoea 1 to zoea 2 was higher in the San Francisco regular, Canada enriched, and hard clam treatments.

KEY WORDS: Broodstock diet, *Lysmata wurdemanni*, ornamental shrimp, reproductive performance

INTRODUCTION

Practically all of the marine organisms marketed in the aquarium trade industry are collected from coral reef ecosystems. Extensive and destructive collection has caused great concerns and much efforts have been devoted to develop technologies to cultivate these species. Peppermint shrimp, *Lysmata wurdemanni*, is one of the most popular species in aquarium trade industry. Complete life-cycle culture of peppermint shrimp has been achieved in the laboratory, using newly hatched *Artemia* nauplii as the only food (unpublished data). However, there are still some obstacles in commercial production of the species. For example, there are variations in larval duration (ranging from 90 to over 100 days); sometimes embryos are aborted prematurely; and some larvae from captive broodstock have difficulty separating from the old exoskeleton during molting (unpublished data). Improving broodstock diet may enhance reproductive performance, thereby increasing the commercial production potential of the species.

Frozen adult *Artemia* can be used as a supplement or replacement of maturation diet for *Penaeus semisulcatus* (Browdy et al. 1989), *P. stylirostris* (Bray et al. 1990), and *P. vannamei* (Naessens et al. 1997). It is possible to boost the *Artemia* with nutritional supplements, such as highly unsaturated fatty acids (HUFAS), which may stimulate reproductive performance in shrimp. Individuals of *Penaeus chinensis* (Xu et al. 1992, 1994) and *P. indicus* (Cahu et al. 1995) fed with broodstock diets containing higher HUFAs produce better eggs with higher hatching rates. The tissue of clams, squid, or shrimp is rich in n-3 HUFA, arachidonic acid, cholesterol and other sterols, phospholipids, and essential amino acids, and appears to promote spawning success in penaeid shrimp (see Harrison, 1997 for a review).

The present study compares the effects of different types of frozen adult *Artemia* and hard clam *Mercenaria mercenaria* as broodstock diets on the reproductive performance of the peppermint shrimp.

MATERIALS AND METHODS

The study was conducted at Florida Institute of Technology, USA from May 1999 to May 2000. Farm-raised adult shrimp used in the experiment were purchased from Oceans Reefs & Aquariums, Inc. at Fort Pierce, Florida and maintained in a recirculating seawater system under 14 h light followed by 10 h dark. Water temperature was controlled at 27–29°C and salinity at 31–33 ppt. The shrimp were fed in excess once a day with one of the five test broodstock diets: four types of frozen adult *Artemia*: regular and enriched with (HUFA, phospholipids, steroids, carotenoids and vitamins) from two sources (Canada and San Francisco Bay); and frozen hard clam (*Mercenaria mercenaria*). Thirty-five shrimp of similar total length (TL, from the end of telson to the tip of rostrum) (Table 1) seven for each treatment, were used.

The shrimp were fed with the test diet for at least one month prior to collecting the following parameters: TL; wet body weight (WBW); fecundity (number of eggs); egg diameter and dry weight; and larval survivorship from Zoea 1 (Z_1) to Zoea 2 (Z_2). Two consecutive spawns from each experimental shrimp were used. Thirty newly hatched larvae (Z_1) were selected from the first spawn and TL of each larva was measured under a dissecting microscope with ocular. Each larva was then placed in a 270-ml transparent plastic cup with 220-ml seawater and fed with newly hatched *Artemia* nauplii. The number of larvae that survived to Z2 (30–36 hrs. later) was counted and survivorship was calculated.

At about 24 h after the second spawning, the entire egg mass was removed gently from the shrimp using small forceps and number of eggs were counted. Diameters of 30 eggs were measured under a microscope with an ocular micrometer. Egg volume was calculated using the formulae $V = 1/6(\pi d^3)$ for spherical eggs (d is the egg diameter) and $V = 1/6(\pi d_1 \times d_2^2)$ for prolate spheroids (d_1 is the least diameter and d_2 is the greatest diameter). One hundred eggs were separated from the rest of the eggs and all were dried at 70°C for 48 h. The total weight of the 100 eggs was measured to the nearest 0.01 mg using a digital balance and mean dry weight per egg was calculated. The spawned shrimp were dried on blotting paper, TL (to the nearest 0.1 mm), and WBW (to the nearest 0.1 mg) were measured.

Five different-sized shrimp (from 2.81 to 4.50 cm in TL) were used to compare the fecundity and egg volume from consecutive spawns. For each shrimp, fecundity and diameter of 30 eggs were measured from each of the two spawns.

One-way ANOVA was used to test the effect of different foods on average (of seven replicate shrimp) fecundity, relative fecundity (fecundity/g WBW), egg volume, dry egg weight, egg density (egg dry weight/egg volume), larval total length and survivorship from

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TABLE 1.

Effect of frozen Artemia and clam as broodstock diet on reproductive performance of the peppermint shrimp, *Lysmata wurdemanni* (mean ± s.d., $n = 7$)

	San Francisco Bay		Canada			
	Enriched	Regular	Enriched	Regular	Clam	ANOVA
Adult total length (cm)	3.84 ± 0.24	4.17 ± 0.29	3.95 ± 0.51	3.98 ± 0.30	3.84 ± 0.15	ns
Fecundity (number of eggs)	1267 ± 322	1456 ± 429	1203 ± 261	1080 ± 340	1092 ± 224	ns
Relative fecundity (#/g female)	1578 ± 241	1309 ± 145	1291 ± 124	1357 ± 139	1304 ± 274	ns
Egg volume (mm^3)	0.0582 ± 0.0049^b	0.0639 ± 0.0064^a	0.0565 ± 0.0032^b	0.0582 ± 0.0029^b	0.0589 ± 0.0034^b	**
Egg dry weight (μg/egg)	30.0 ± 2.3	33.0 ± 3.1	30.9 ± 3.1	30.9 ± 2.4	30.4 ± 2.0	ns
Egg percent ash	2.5 ± 1.2	2.3 ± 0.9	2.4 ± 0.8	2.4 ± 0.7	2.4 ± 1.0	ns
Larval total length (mm)	2.57 ± 0.06	2.55 ± 0.05	2.51 ± 0.03	2.57 ± 0.05	2.56 ± 0.96	ns
Survivorship from Z_1 to Z_2 (%)	75.5 ± 10.2	87.6 ± 15.9	88.1 ± 9.2	71.9 ± 13.7	86.7 ± 4.7	*

* Significantly different ($P < 0.05$).

** Highly significantly different ($P < 0.01$).

ns, Not significantly different ($P > 0.05$).

Z_1 to Z_2, respectively, among the treatments. If the ANOVA result was significant, Fisher LSD method was employed to test for differences among the means. A two-tailed Student's *t*-test was used to compare the average fecundity and egg volume between the two consecutive spawns.

RESULTS

There was no significant difference in female total length among the five treatments (Table 1). Of the seven reproductive parameters measured, only egg volume and survivorship from Z_1 to Z_2 show significant differences among the treatments (Table 1). Egg volume from the regular San Francisco brand *Artemia* is significantly ($P < 0.01$) larger than those of the other treatments that do not significantly different from one another. The survivorship from Z_1 to Z_2 is significantly higher in the Canada enriched, San Francisco regular, and hard clam treatments than in the San Francisco enriched and Canada regular treatments (Table 1). There is no significant difference in fecundity ($P = 0.89$) between the first (mean ± s.d. = 953 ± 614) and the second (mean ± s.d. = 898 ± 464) spawns. Similarly, the difference in average egg volume between the first (mean ± s.d. = 0.0593 ± 0.0074 mm^3) and second (mean ± s.d. = 0.0533 ± 0.0074 mm^3) spawns is not statistically significant ($P = 0.23$).

DISCUSSION

Peppermint shrimp, also called red cleaning shrimp, may feed on parasites of fish in its natural environment, like many of its sibling species in the genus (Limbaugh et al. 1961, Debelius 1984, Jonasson 1987). Therefore, its natural diet may be narrow (although little is known on the relative importance and composition of parasites in the shrimp's natural diet). We found no significant difference in five of the seven reproductive performance parameters measured among the five-broodstock diets tested. There is no clear pattern in the two parameters (egg volume and survivorship from Z_1 to Z_2) showing consistent differences among the treatments. Larger eggs in the San Francisco regular treatment may be the result of larger (though not statistically significant) total length of the females, as egg volume correlates positively with maternal size in *Lysmata wurdemanni* (Lin and Zhang, unpublished data). In a sibling species, *L. debelius*, larval production is higher for those fed with live *Artemia* nauplii and those with enriched (with HUFAS, vitamins and others) frozen *Artemia* nauplii than those fed with regular frozen *Artemia* nauplii, which in turn is significantly and substantially (about 100%) higher than those fed with natural diets (mixture of fresh mussel and polychaete) (F. Simoes, pers. comm., 1998). However, in the same study on another sibling species, *L. amboinensis*, enriched frozen *Artemia* nauplii and natural diet resulted in the same level of larval production (F. Simoes, pers. comm. 1998). Browdy et al. (1989) demonstrated that using frozen adult *Artemia* as a dietary supplement can enhance reproductive performance of *Penaeus semisulcatus*. The reproductive activity was not consistent, however, and this may be due to possible variability in the nutritional quality of the different batches of *Artemia* used. The enrichment of *Artemia* is supposed to increase nutritional quality and reduce variability among *Artemia* batches. Naessens et al. (1997) showed that frozen *Artemia* may be useful as a supplement or replacement for polychaetes as maturation diet for *Penaeus vannamei*. However, brine shrimp biomass enriched with HUFAS did not perform better than the regular *Artemia* in their experiments. The authors caution the interpretation of the results and suggest that more research is needed to compare enriched and regular brine shrimp biomass and identify potentially active ingredients and their optimal doses.

ACKNOWLEGMENTS

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THE TEXAS LIVE BAIT SHRIMP MARKET

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ABSTRACT Decreases in wild catches coupled with increased regulations have attracted interest in the production of farm-raised live bait shrimp. Recent refinements in penaeid culture techniques suggest that the production of live bait is a potentially profitable endeavor. In this study, live bait shrimp dealers in Texas were surveyed by mail. The return rate was 8%. Returned surveys were found to be from 33 businesses from various locations over the entire Texas Coast. The information collected provides a small database that can be used to provide a broad and general view of the Texas market demand for farm-raised live bait shrimp. Texas market dynamics were characterized in terms of regional demands and seasonal fluctuations. Most live bait retail suppliers in Texas have been operating for 6–20 years, and during any given year they are able to meet the market demands for two only months. Most retailers expressed acceptance of a farm-raised product if it was of good quality and consistently supplied.

KEY WORDS: Texas, Gulf of Mexico, live bait shrimp, *Farfantepenaeus aztecus*, *F. duorarum*, *Litopenaeus setiferus*, survey

Historically, the supply of live bait shrimp for the eastern Atlantic and Gulf of Mexico coasts has relied on the harvest of wild *Farfantepenaeus aztecus* (Atlantic brown shrimp), *F. duorarum* (Atlantic pink shrimp), and *Litopenaeus setiferus* (Atlantic white shrimp). The overharvest of this natural resource forced the National Marine Fisheries Service to take action to maintain a sustainable yield of wild-caught shrimp (Warren 1980, McKee 1986, Southern Fisheries Science Center [SFSC] 1992, Nance 1993). The status of the Gulf of Mexico shrimp fishery for *F. aztecus*, *F. duorarum*, and *L. setiferus* was declared overexploited by the National Marine Fisheries Service in 1990 (SFSC 1992). The catch fishery is currently seen as overcapitalized, with more effort expended per unit catch than is economically feasible (SFSC 1992). The increasing overexploitation of the wild shrimp fishery has caused the bait shrimp fleet on the Texas coast to redirect its catch emphasis from the historically abundant white shrimp, *L. setiferus*, to the less abundant brown shrimp, *F. aztecus* (Texas Parks and Wildlife Department 1995). The Texas Parks and Wildlife Department's shrimp management proclamation recognizes that the continuing increase in the harvest of small shrimp (>67 count/lb) is ecologically unsustainable and that it will cause shrimp stocks to collapse (Texas Parks and Wildlife Department 1993). The decline in wild harvests along the Texas Coast was observed, as early as 1986, to have an effect on retail markets (McKee 1986). McKee et al. (1989) observed that the retail demand for live bait shrimp in Texas waters was greater than what the bait shrimp fleet was able to supply.

The status of the fishery prompted the establishment of a Gulf of Mexico management plan and prompted the South Atlantic Management Council in 1981 to regulate the fishery along the Gulf of Mexico and eastern coast of the United States. Regulations at the state level have also been forthcoming. In 1995, the state of Texas signed into law HB750, which imposed new regulations on bait shrimpers. This bill was designed to provide long-term conservation of shrimp stocks and to create economic stability in the bait shrimp fishery (Texas Parks and Wildlife 1995). For example, the bill limits the number of future bay bait shrimping licenses, which will eventually lead to a reduction in the bait fishing fleet.

Due to the great demand and high dollar value of live bait shrimp, the production of farm-raised live bait shrimp has attracted much attention in the past few years. Recent studies have reported on the profitability of producing cultured live bait shrimp using modern farming techniques (McKee 1986). Limited data were available concerning the economic viability of a live bait shrimp farm (Parker & Holcomb 1973, Quick & Morris 1976, Rossberg & Strawn 1980). The initial research concluded that farming live bait shrimp was unprofitable (McKee et al. 1989). However, recent studies suggest that the production of live bait shrimp is a potentially profitable endeavor (Sandifer et al. 1993, Burkott 1994, Samocha et al. 1998). These studies have focused on the manipulation of stocking density, the development of new culture techniques, and pond management to improve the profitability of live bait shrimp production.

Past studies concerning Texas' demand for live bait shrimp relied on National Marine Fisheries Service landing reports and personal interviews in Texas (McKee 1986, Burkott 1994). These studies provided only a partial estimation of consumption, production volume, and a general pricing structure of this market because data were collected mainly from local markets. Assessment of the true potential of a live bait shrimp farming requires an understanding of production and infrastructure costs, market demand, and market price data for the major regions of this market.

The objectives of the current study were (1) to collect information concerning the live bait shrimp business type and market environment in Texas, (2) to analyze the supply and demand relationships of live bait shrimp within this region; and (3) to assess the acceptability of farm-raised live bait shrimp by retailers.

A mail-out survey was used to collect data on the regional live bait shrimp market demand and supply. In general, the data characterize seasonal fluctuations in the supply and demand of bait shrimp. In addition, this survey provides an overall view of the variation in the Texas wholesale pricing structures, quantity demand, mortality rates of wild-caught live bait shrimp, average quantity demand of each retail outlet, and acceptance of farm-raised bait shrimp by retailers. These data also provide an understanding of regional quantity demand, species usage, and the size of live bait shrimp preferred by this market.

MATERIALS AND METHODS

The research was conducted at the Texas Agricultural Experiment Station Shrimp Mariculture Research Facility in Corpus

Christi, Texas. Mailer information of live bait dealers was obtained from the Texas Parks and Wildlife Department. Survey forms were mailed to the 412 licensed live bait dealers in Texas. The survey contained 31 multiple-choice questions and two short-answer questions. Questions were presented in an easy-to-read format. The surveys were mailed between July 25, 1996 and August 5, 1996 with a self-addressed stamped return envelope to improve the rate of return.

RESULTS AND DISCUSSION

Of the 412 licensed Texas bait dealers, 33 returned the survey (8% return rate). The low return rate of this survey suggests some inherent bias due to the use of a small data set. The return data, when grouped by physical location along the Texas coast, shows that the returned surveys were not from one specific area. These surveys were in fact from 33 businesses from various locations over the entire Texas Coast. From this data set, which is limited in volume but broad in scope, it is possible to understand the Texas market from a general perspective.

The Texas live bait shrimp retail market is comprised of retailers who have been operating in this market for 6–20 y (Fig. 1). Texas retailers indicated an increase in live bait shrimp demand over the past 5 y without an increase in supply. The inability of the bait shrimpers to supply the Texas market, observed in the early years by McKee (1986), appears to continue today. Texas retailers indicated that a general lack of supply is expected nearly all year round. Texas retailers expect a semi-consistent supply of live bait shrimp in May, June, and October; however, this supply is not reliable (Fig. 2), although the highest demand for live bait shrimp by fishermen lasts from April to October (Fig. 2). The primary limiting factor in meeting the demand is the bait shrimping laws that limit catches to 200 lb/vessel/day (TPWD 1995).

The sales of live bait shrimp accounted for more than 50% of the sales for 29% of the live bait dealers. Another 29% of the dealers reported live bait shrimp sales that accounted for 25–49% of their total sales (Fig. 3). Live bait shrimp is the key to success for all bait and tackle stores. Lack of live bait also effects the sales of other merchandise in a bait and tackle store.

In Texas, the holding capacities are limited to 22–45 kg (50–100 lb.) of live shrimp per retailer. The retailers are restocked daily during the high-demand periods and daily to weekly during low-demand periods. Under the high demand for bait shrimp in this market, supply becomes an important factor. Ninety-four percent of the retailers surveyed responded that three local suppliers supply them; only a small portion of the Texas supply is imported from Florida.

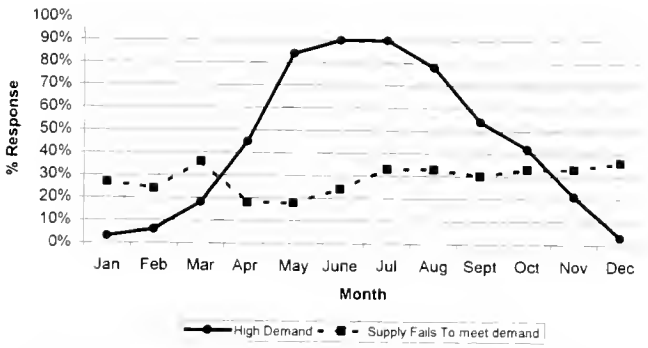

Figure 2. Consumer demand vs. availability for Texas live bait shrimp market.

The controlling factors involved in the selection of a supplier by Texas retailers are availability and consistency. The ability of a farm-raised product to meet these criteria illustrates its advantage over the wild-caught live bait shrimp. When asked if they would purchase a farm-raised product, 74% of the retailers replied positively, provided the supply is consistent and of good quality (e.g., hardy animals with minimal signs of broken appendages and lesions).

This survey showed that 36% of the live bait shrimp dealers in Texas listed *F. aztecus* as their species of choice, 24% preferred *F. duorarum*, 21% had no preference, and 15% preferred *L. setiferus*. The data further corroborates the earlier study by McKee (1986), which suggested that sport fishermen prefer brown shrimp due to its greater availability and high tolerance to stress.

The current study further suggests that the majority of the live bait shrimp are purchased by the gallon (3.785 l) with a size preference of 60–70 count/lb (132–154/kg) or individual shrimp weight between 6.5 and 7.6 g. The average price for this size of shrimp was $21.23/g. Inglis and Chin (1966) reported a retailer preference for 80-count/lb shrimp (5.76 g per shrimp). On the other hand, Quick and Morris (1976) suggested a preference for 200-count shrimp (2.27 g per shrimp). Our data corroborates the wholesale and retail pricing trends suggested by earlier studies.

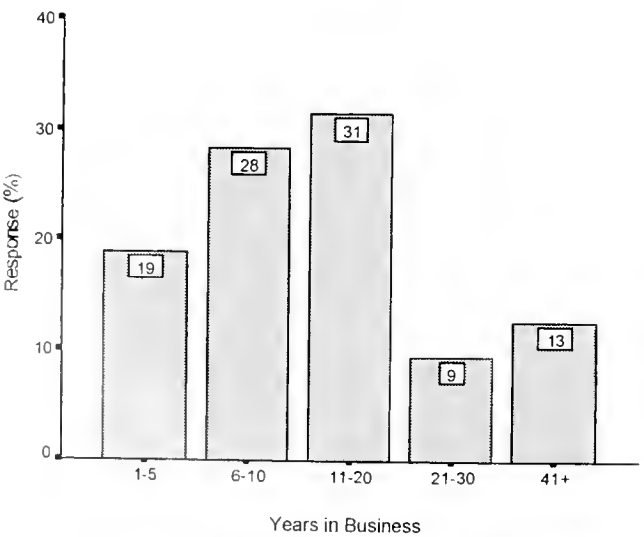

Figure 1. Texas live bait shrimp retail business age.

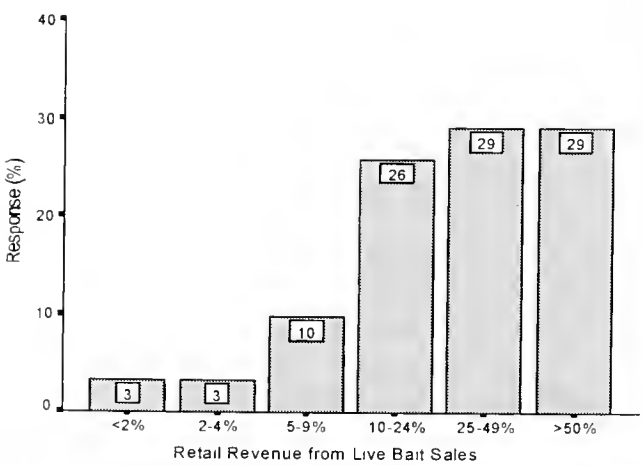

Figure 3. Percent revenue expected by retailers from sales of live bait shrimp.

The wholesale price for bait shrimp has been documented to fluctuate according to supply, whereas the retail price remains constant within a region (McKee 1986).

Prior studies have reported an accepted mortality of 10–20% for live bait shrimp after delivery (McKee 1986). This survey found the expected mortality to be higher. Forty percent of the retailers expected the standard 10–24% mortality per shipment, whereas 20% expected 25–49% mortality (Fig. 4). This great loss of stock translates directly into lower revenues. Although lack of stock has an impact on sales, the weather was cited as the primary controlling factor that negatively impacts retail sales.

This study has concluded that, in general, the Texas market for live bait shrimp is largely undersupplied during the highest demand periods of the year. Whether this is due to a declining resource or due to tighter catch restrictions is a point of contention. However, the fact remains that this market has a considerable lack of supply. Live bait shrimp sales account for a majority of retailers' yearly revenue. This is due to the ability of live bait shrimp to drive the sales of other items in bait stores (e.g., equipment and food) if it is available. Thus, it is easy to see that the market is not realizing its full potential without a consistently available and reliable product. The acceptance of a farm-raised live bait shrimp, with its flexible product characteristics, would allow it to meet the major demands of the Texas market.

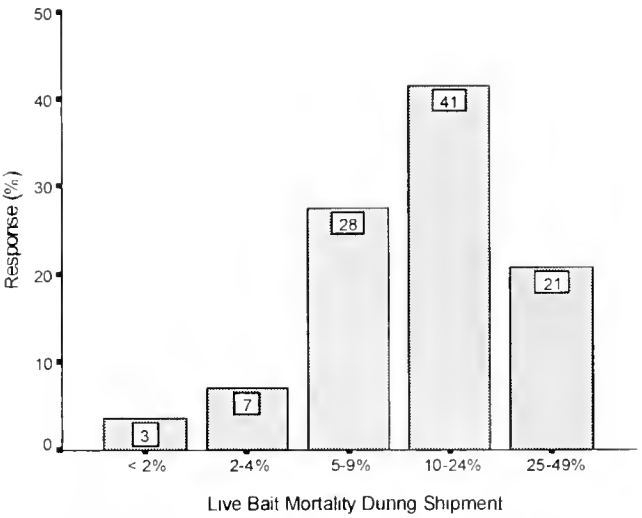

Figure 4. Expected live bait shrimp mortality during shipment.

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THE REPRODUCTIVE CYCLE OF GOLDEN KING CRAB *LITHODES AEQUISPINUS* (ANOMURA: LITHODIDAE)

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ABSTRACT Female golden king crabs *Lithodes aequispinus* were held in captivity to determine the duration of their reproductive cycle. The time between the first and last egg hatching was 34 days (SD = 16, n = 147) on average. Females molted about 192 days (SD = 72, n = 111) after the last egg hatched. Eggs were extruded 2 days (SD = 2, n = 61) after molting. Egg clutches were incubated for an average of 362 days (SD = 78, n = 59). The average amount of time that passed between the production of successive egg clutches was 590 days, or 3,570- degree days of water temperature.

KEY WORDS: *Lithodes aequispinus*, reproduction, king crab

INTRODUCTION

The golden king crab, *Lithodes aequispinus* Benedict, supports an important fishery in Alaska. This North Pacific species typically lives in deep water, on untrawlable bottoms. Because of their remote deep habitat, and the fact that only males are retained by the fishery, the reproductive cycle of females had not been described in detail prior to this study. Female golden king crabs carry up to 27,000 large eggs (Jewett et al. 1985) that can hatch in any month of the year (Adams and Paul 1999). Female golden king crabs are found in all reproductive and molting stages throughout the year (Paul and Paul 2000a), so the duration between clutches was not apparent from gross examination of the egg mass. This laboratory study examined the reproductive cycle of the golden king crab to identify how long it took females to go from the extrusion of one egg clutch to another. This study was accompanied by parallel studies on growth of both sexes (Paul and Paul 2000a) and sizes at maturity of males (Paul and Paul 2000b).

MATERIALS AND METHODS

Crabs needed for the study were captured with pots fished at 108 to 152 m depths on the western side of Prince William Sound, Alaska. After capture the specimens were transported to the Seward Marine Center Laboratory by floatplane. No mortalities occurred during the transport process. Collections of multiparous females were made November 11–14,1996; May 1, 1997 and October 10–20, 1998. Observation of captives continued until October 1, 2000.

The carapace length (CL) of all crabs was measured to the nearest millimeter for the growth study (Paul and Paul 2000a), and they were tagged with a numbered plastic disk attached to the leg with a plastic cable tie. All females in this study had egg clutches when captured and they had carapace lengths ranging from 104 to 150 mm ($\bar{x}$ = 132, SD = 7 mm). Each female was held in a separate 800 to 1000 L tank. The seawater in the tanks was exchanged ≥100% per hour to maintain the ambient temperature of incoming water from 75 m depth in Resurrection Bay. The water temperature in the tanks was measured daily. The duration of each of the 4 reproductive phases was described as both, number of days and degree-days of water temperature. The degree-day intermolt period is considered the sum of the average daily temperature during the intermolt period. Thus for example, if a stage interval was 30 days and the temperature every day was 6°C, the event spanned 180-degree days (30 × 6). The degree-day duration data was calculated because the reproductive phases were nonsynchronous with some females extruding new clutches in the warm part of the year and others during the cold season. Temperature in the tanks followed the fjord's seasonal cycles ranging from 3.7 to 9.7°C (Fig. 1). Crabs were fed every other day to excess with a repeating cycle of the following foods: whole Pacific herring *Clupea pallasi* Valenciennes 1847, fillet of coho salmon *Oncorhynchus kisutch* (Walbaum 1792), giant Pacific Octopus *Octopus dofleini* (Wulker 1910), whole squid (species unknown), and whole Alaska northern shrimp *Pandalus eous* Makarov 1995.

STAGE 1 (n = 147) was the length of time between the hatching of the first and last egg. STAGE 2 (n = 111) started after the last egg hatched and it ended when a female molted. STAGE 3 (n = 61) was the length of time between the day a female molted and the day she extruded a new egg clutch. The duration of clutch incubation (STAGE 4) was the length of time between the extrusion of a new egg clutch and the hatching of all those eggs (n = 59). Thus, STAGE 1 and 4 overlap. The duration of the different phases of the full reproductive cycle was not quantified for every female since captives had different collection dates.

Tanks were examined daily for the presence of larvae and when the first larvae were seen, females were examined every day until all eggs had hatched. This procedure was used to determine the duration of the larval hatching phase, STAGE 1. Thereafter, females were isolated from males until they molted. Females must molt prior to mating. These observations estimated the time between egg hatching and molting, STAGE 2.

After a female molted a selected hardshell male ≥114 mm CL was put into the tank with her. Males of this size are capable of fertilizing females (Paul and Paul 2000b). The time between the molt and the occurrence of the new egg clutch was termed STAGE 3. After ovulation, females were isolated and held until zygotes developed to the 64-cell stage. Groups of at least 100 eggs from each pleopod were then randomly selected and examined under a microscope for cell division to determine percent viability. Females were held from ovulation until hatching to determine the incubation period, STAGE 4.

RESULTS

STAGE 1, the period between the first and last larvae hatching, averaged 34 days (SD = 16, n = 147, range 8–85) or 202 degree days (SD = 88, range 19–474). On average 192 days (SD = 72,

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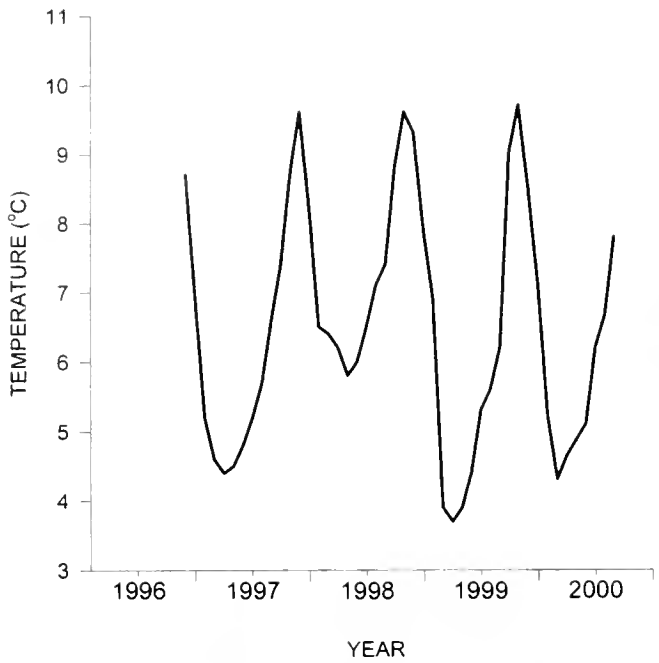

Figure 1. Laboratory seawater water temperatures during the study.

n = 111, range 5–464) or 1,084 degree days (SD = 428, range 36–2,762) passed after the last larvae hatched until the females molted which completed STAGE 2. During STAGE 3 females typically extruded new eggs 2 days (SD = 2, n = 61, range 1–12) or 15 degree days (SD = 12, range 4–85) after molting. All clutches resulting from these laboratory matings had ≥80 of their eggs initiating division. STAGE 4, egg clutch brooding, lasted an average of 362 days (SD = 78, n = 59, range 40–569) or 2,269 degree days (SD = 570, range 114–2,754). The total time passing between egg clutches averaged 590 days or 3,570 degree days. The best fitting regressions relating the CL (mm) of all females that completed STAGES 1–4 and their reproductive cycle duration (degree days) showed no apparent relationship between these variables ($r^2 \leq 0.12$).

DISCUSSION

In early studies the time of spawning of golden king crab was described both as seasonal asynchronous and synchronous (Sloan 1985). Some of this reported variability may have been caused by an imperfect understanding of the reproductive cycle. During STAGE 2 ($\bar{x}$ = 192 days) females carry decaying empty egg capsules on their setae (Sloan 1985) and this condition made it difficult for fishery observers to classify the reproductive status of females.

For Prince William Sound golden king crab, molting and hatching events can occur in any month in captive females (Paul & Paul 1999, Paul & Paul 2000a). Thus, their reproductive cycle is markedly different from the genus *Paralithodes*. In red king crab *Paralithodes camtschaticus* (Tilesius) the female reproductive cycle is synchronous, lasting about one year (Paul & Paul 1990, Paul & Paul 1997). In blue king crab (*Paralithodes platypus* Brandt) primiparous females may produce egg clutches annually and every two years for larger multiparous females. Like red king crabs, the eggs of primiparous and multiparous blue king crab hatch during the spring plankton bloom (Jensen & Armstrong 1989). Red king crabs hatch their eggs in spring so the larvae can feed on the plankton bloom (Paul et al. 1990), then all ripe females molt and breed soon after hatching is done. With golden king crab, hatching does not need to occur exclusively during the spring plankton bloom because their lecithotropic larvae do not feed and they can tolerate both summer and winter temperatures (Shirley & Zhou 1997, Adams & Paul 1999, Paul & Paul 1999). One striking difference between the reproductive cycle of golden and red king crabs is the amount of time between egg hatching and the female molt. In red king crabs captive females usually molt within two weeks of egg hatching (authors' unpublished observations) vs. about six months for golden females. Currently it is not known why these differences exist. Golden king crab females may need more time to produce the large yolk rich eggs that allow their larvae to forgo feeding. However, that idea is speculation at this time.

Female Prince William Sound golden king crab first mature around 120 mm CL and typically grow to about 150 mm CL in 5 molts (Paul & Paul 2000a). If they produced an egg clutch every molt, and their reproductive cycles lasted 590 days, females would have to survive at least eight years to produce 5 clutches.

The sea water for holding the crabs came from a 75 m depth where temperatures during August to December are 1 to 4°C warmer than at approximately 150 m where the specimens were captured, versus 1 to 2°C cooler from January to April (Xiong & Royer 1984). Generally warmer conditions decrease intermolt duration in crustaceans unless there is thermally induced stress and cooler ones lengthen it. Only *in situ* studies, or laboratory studies that mimic site specific temperatures, can determine if our degree day estimates for the reproductive cycle duration are appropriate for female golden king crab living in different thermal environments.

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INTERMOLT DURATIONS OF CAPTIVE JUVENILE AND ADOLESCENT MALE TANNER CRABS *CHIONOECETES BAIRDI*

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ABSTRACT This project examined the effect of temperature on intermolt durations of juvenile and adolescent male *Chionoecetes bairdi* Rathbun with carapace widths (CW) of 11 to 105 mm. Juveniles were identified by the absence of spermatophores and adolescents by their presence. The relationship between male maximum CW and intermolt duration exhibited a linear relationship for juveniles described by the equation: Intermolt period (degree-days) = 7.9 (CW mm) + 272; $r^2 = 0.53$. As males began to produce spermatophores at about 55 mm CW intermolt durations increased and the analogous equation was Intermolt period (degree-days) = 7.6 (CW mm) + 1,215; $r^2 = 0.14$. A 55 mm CW spermatophore bearing male took about 2.5 times longer to molt than a similar sized juvenile.

KEY WORDS: *Chionoecetes*, molting, temperature, tanner crab

INTRODUCTION

In the northern Gulf of Alaska and the southeastern Bering Sea the Tanner crab, *Chionoecetes bairdi*, is a ubiquitous benthic invertebrate that is harvested commercially. The change in carapace size following molting has been described for Gulf of Alaska *C. bairdi* (Donaldson et al. 1981, Paul & Paul 1996) but the intermolt duration has not. Information on intermolt duration is critical to understanding recruitment to the population because there is no accepted method to age Tanner crabs (Rosenkranz et al. 1998). Crustacean growth rates are controlled by a number of factors with temperature being one of the most obvious (Fisher 1999). Benthic temperatures in the Gulf of Alaska experience long term warming and cooling cycles of about 18 to 20 years (Royer 1989). In addition to seasonal changes in temperature El Niño events, like that of 1998 (Fig. 1), are additional sources of thermal perturbations. The inter-annual differences in the mean monthly bottom temperature where the specimens for this study were captured were on order of 2 to 3°C (Fig. 1). Currently there is no information on the effect warm and cold periods have on the duration of molting rates of *C. bairdi*. It is logical to assume that thermal conditions will be a major factor regulating the length of the intermolt period. The objective of this study was to determine the intermolt period of juvenile and adolescent male Tanner crabs in degree-days. This study was funded to improve our understanding of the growth process and the relative age of males recruiting to adulthood. In earlier observations of size of maturity and growth in male Tanner crabs (Adams & Paul, 1983; Paul & Paul, 1996) it appeared that immature males took less time to molt than mature males of similar sizes but this was not quantifiable with the methods used. We speculated that the molting schedules of mature and immature males would be markedly dissimilar, so we ascertained the maturity status of test specimens to examine this theory.

MATERIALS AND METHODS

Male Tanner crabs were collected at the head of Resurrection Bay near Seward Alaska at 35 to 80 m depth using a 2 m otter trawl with 6 mm cod end mesh at a variety of times during the years 1996 to 2000. There were 67 males with carapace widths (CW) 9 to 82 mm that were returned to the nearby laboratory for the study. The seawater for the Seward Laboratory comes from 75-m depth in a fjord and its temperature during the study was 3 to 10°C (Fig. 1). The temperature of the incoming water changes with season with marked inter-annual variations in monthly values (Fig. 1). Each day the seawater temperature in the tanks was recorded. Salinity ranged from 31 to 33 ppt. All test animals were held in separate numbered tanks to prevent cannibalism. Males ≤45 mm were held in individual 20-L tanks. Males ≥46 mm were held in 100-L tanks and the water exchange rate in all tanks was 100% per h.

Captives were fed to excess every Monday (whole northern shrimp *Pandalus eous* Makarov 1995), Wednesday (live intertidal mussel *Mytilus trossulus* Gould 1850) and Friday (Coho salmon fillet *Oncorhynchus kisutch* Walbaum 1792). Whenever a male molted for the first time in captivity the date of molting was recorded. After two weeks had passed, and the carapace had hardened, its maximum CW was measured to the nearest 0.1 mm. The new post molt CW of the smallest captive was 11 mm and the largest 105 mm CW. A three millimeter coded plastic disk was glued to the carapace of test specimens. The date of each crab's second molt was recorded to calculate the intermolt duration. The new CW was measured two weeks later and the vas deferens was removed to determine if spermatophores were present in wet mounts using 100× magnification.

The intermolt duration was described in degree-days. Degree-days were calculated by summing the daily seawater temperatures that occurred during the intermolt period. For example if an event took ten days, and each day the temperature measurement was 10°C, then the process would have taken 100 degree days.

RESULTS

The relationship between male CW and intermolt duration exhibited a linear relationship for juveniles that was described by the equation: Intermolt period (degree-days) = 7.9 (CW mm) + 272; $r^2 = 0.53$, $P < 0.0001$, $n = 53$ (Fig. 2A). The relationship between CW (mm) and intermolt duration for males producing spermatophores was described by the equation: Intermolt period (degree-days) = 7.6 (CW mm) + 1,215; $r^2 = 0.14$, $P = 0.1832$, $n = 14$ (Fig. 2B). The intermolt duration of mature males was less dependent on temperature than that of juveniles. The intermolt durations of males that were producing spermatophores (Fig. 2B, C) were longer than would be expected if the linear models of intermolt

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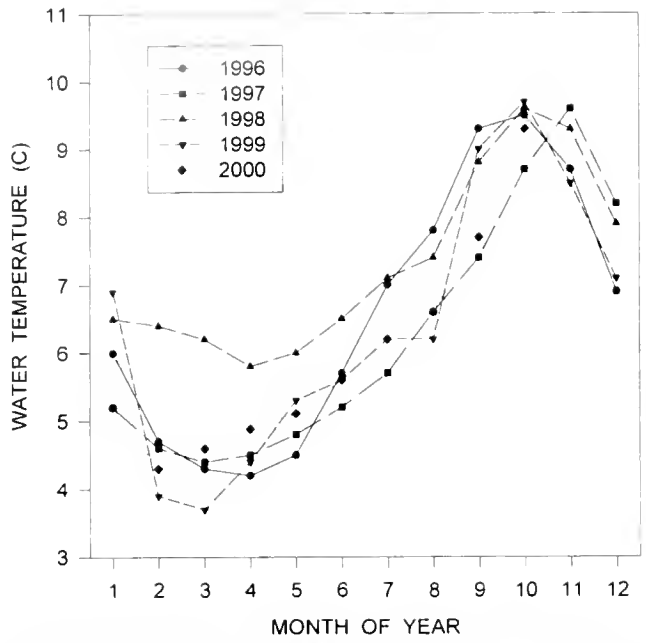

Figure 1. Seawater temperatures at 75 m depth in Resurrection Bay, near Seward Alaska between 1996 and 2000.

duration for juvenile individuals (Fig. 2A) and adolescents (Fig. 2B) coincided (Fig. 2C). For example, the largest male without spermatophores was 56 mm CW and it had an intermolt duration of 672 degree-days (Fig. 2C). The smallest male with spermatophores was 55 mm CW and 1,692 degree-days passed before it molted (Fig. 2C).

Based on the equation describing the intermolt duration (Fig. 2A), and using the growth per molt equation of: New CW = Initial CW (1.14) + 4.1; $r^2 = 0.97$ (Paul and Paul 1996) we estimate that a recently metamorphosed Tanner crab of 3 mm CW would require approximately 3,639 degree days to reach 63 mm CW (Table 1), a size when >50% of Gulf of Alaska males have spermatophores (Paul 1992). A spermatophore bearing male 63 mm CW would require about 7,414 degree-days to grow to 107 mm CW (Table 2), a size close to that of the largest individual that molted in this study. The largest male in the study was 105 mm CW and it molted after 2,001degree-days. The smallest male in the study was 11 mm CW and it molted after 477 degree-days.

DISCUSSION

It is generally accepted that the amount of time that passes between molts increases with crab carapace size, and this proved to be true for *C. bairdi*. We are not sure why small spermatophore bearing males had longer intermolt periods than similar sized im-

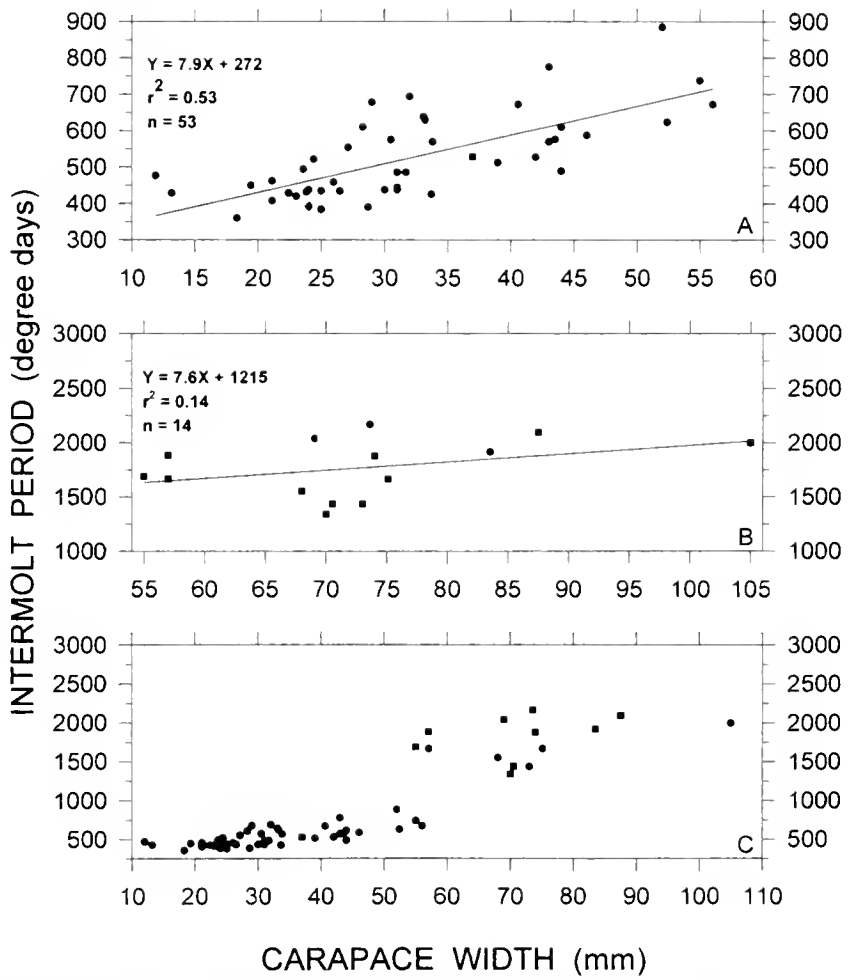

Figure 2. Intermolt durations in degrees days for juvenile male *Chionocetes bairdi* that did not have spermatophores in their vas deferens (Panel A, ●), spermatophore bearing males (Panel B, ■), and comparisons of both types (Panel C).

TABLE 1.

The estimated intermolt durations in degree days for male juvenile *Chionoecetes bairdi* relative to carapace width (CW).

Initial CW (mm)[a]	New CW (mm)[a]	Intermold duration (degree days)[b]	Σ Degree days
3.0	7.5	276	276
7.5	12.6	313	598
12.6	18.5	358	947
18.5	25.2	408	1355
25.2	32.8	464	1819
32.8	41.6	529	2348
41.6	51.5	603	2951
51.5	62.8	688	3639

[a]Growth equation from Paul and Paul 1996.
[b]From equations in Figure 2A.

TABLE 2.

The estimated intermolt durations in degree days for spermatophore producing male *Chionoecetes bairdi* relative to carapace width (CW).

Initial CW (mm)[a]	New CW (mm)[a]	Intermolt duration (degree days)[b]	Σ Degree days
62.8	75.7	1692	1692
75.7	90.4	1790	3482
90.4	107.2	1902	5384
107.2	126.2	2030	7414

[a]Growth equation from Paul and Paul 1996.
[b]From equations in Figure 2B.

mature males. Perhaps spermatophore production is only one element of a complex maturation process with morphological, biochemical and physiological changes that involves longer intermolt periods. Currently, no other information on the intermolt durations of juvenile Tanner crabs, or closely related high latitude species, is available to compare to our results. The smallest Tanner crab we have seen in trawl samples is 3 mm CW. Our intermolt duration projections (Tables 1, 2) imply that captive Tanner crabs would grow from 3 to 107 mm CW in 11,053 degree-days. In our laboratory the average annual seawater temperature is about 6° C, and a male held at 6°C would require approximately 1842 days or 5 years to grow from 3 to 107 mm CW. However, we do not know if laboratory conditions, for example tank size, modified molting schedules. In the laboratory a surplus of food was present while in nature food availability may limit growth rates. It is also possible that the laboratory diet was nutritionally incomplete. This study needs replication with *in situ* tagging studies to determine if our laboratory molting schedules are applicable to natural conditions.

These observations on intermolt durations demonstrate that size and maturity status are important factors to consider when forecasting molting schedules in Tanner crabs <105 mm CW. Tanner crab males are not harvested until they are ≥140 mm CW and they can grow to ≈170 mm CW. Thus, molting rate studies need to be done with larger specimens than we used in this observation. Additional experimentation with large crabs would be especially important if there are maturation processes other than spermatophore production that influence molting schedules. Juvenile and adolescent males typically have relatively small claws while fully mature males have large claws (Stevens et al.1993). All the males in this study were small claw morphotypes using the criteria of Stevens et al. (1993), the ratio of chela height/CW <0.17, and most intermolt durations were one year or less. Males continue to molt after reaching maturity (Paul and Paul 1995) presumably because big large-claw males win competitions for mates, and compromised carapaces need replacement. The large claw characteristic develops when males reach 100 to 130 mm CW (Stevens et al. 1993). We studied the consequence of spermatophore presence on molting rates, but not the change to the large claw morphotype, or the attainment of near maximum CW. In another investigation Tanner crabs with CW ≥110 mm had to be held for over two years before they molted (Paul and Paul 1995). None of them were soft-shelled when they were captured, so their intermolt period was longer than two years. In one *in situ* study 47% of tagged male *C. bairdi* > 110 mm CW were recaptured after two years and another 7% after three years (Donaldson 1980). These observations (Donaldson 1980, Paul and Paul 1995) suggest that intermolt durations increase after males assume the large claw morphotype and approach maximum size. Further growth rate studies with males 105 to 170 mm CW are needed to describe the intermolt durations of these large individuals.

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EFFECT OF ARTIFICIAL DIETS CONTAINING CAROTENOID-RICH MICROALGAE ON GONAD GROWTH AND COLOR IN THE SEA URCHIN *PSAMMECHINUS MILIARIS* (GMELIN)

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ABSTRACT Gonadal growth and color were examined in the echinoid *Psammechinus miliaris* when fed diets containing either micro- or macroalgal supplements. Urchins receiving diets containing the microalgae *Phaeodactylum tricornutum* and Tahitian *Isochrysis* sp. showed significantly greater gonad growth at the end of the 12-wk experimental period as compared to urchins fed the artificial diet with no added algae. An improvement in gonad color, compared to the control, was observed for both treatments receiving microalgae; whereas, those fed the macroalgae *Laminaria saccharina* diet showed no significant improvement in color. The *P. tricornutum* diet improved gonad color more rapidly than the other algal diets. These results show that cultured microalgae, incorporated into an artificial diet, have a positive effect on the gonad color of *P. miliaris*, with promising implications for commercial echinoculture.

KEY WORDS: sea urchin, *Psammechinus miliaris*, artificial diet, carotenoid pigments, gonad color, echinoculture

INTRODUCTION

The gonads of sea urchins (Echinodermata, Echinoidea) are a highly sought-after product in many parts of the world, particularly in the Far East and Mediterranean Europe (Hagen 1996). The global demand for sea urchins has risen sharply over the past two decades, and as a result, most commercial sea urchin fisheries are now considered to be fully or overexploited (Keesing and Hall 1998). Attention is, therefore, turning toward the development of a commercially viable echinoculture industry.

Artificial diets have been shown to enhance gonad growth effectively in several commercially important sea urchin species (de Jong-Westman et al. 1995, Lawrence et al. 1997, Barker et al. 1998, Robinson & Colbourne 1998, Fernandez & Pergent 1998). However, in addition to an acceptable quantity of gonad per individual, the marketplace also requires the gonad to be a bright orange color. Addressing the problem of variability in gonad color is, therefore, an important part of artificial diet design.

Carotenoid pigments, the source of red, orange, and yellow coloration in plants and animals are synthesized only by plants and micro-organisms. Animals can, however, alter these molecules by oxidation. The coloration of urchin gonads is a result of selective accumulation and chemical modification of carotenoid pigments obtained from their diet (Goodwin 1984). Griffiths & Perrot (1976) found the primary carotenoids in the ovaries of *Strongylocentrotus droebachiensis* were echinenone (79 – 85%), β-carotene, zeaxanthin, isocryptoxanthin, and small amounts of a fucoxanthin isomer. Echinenone was subsequently shown to be the primary carotenoid in the gonads of 11 species of urchin from Japanese waters (Tsushima & Matsuno 1990) and of the New Zealand echinoid *Evechinus chloroticus* (Goebel & Barker 1998).

Hallenstvet et al., (1978) described the total purified carotenoid content of whole *P. miliaris* as fucoxanthinol 68%, echinenone 10%, lutein 8%, β-β carotene 10% and β-ε carotene 5%, but did not report the relative amount of gonad tissue present or the color of the gonads of the urchins they analyzed. Griffiths and Perrot (1976) found that gut wall of S. *droebachiensis* contained 7–24 times the carotenoid content of the ovaries on a dry weight basis, but that there was little evidence to suggest that fucoxanthin, the main carotenoid of the gut wall, was used in the production of echinenone, the main pigment of the ovary. They presented evidence that echinenone was a conversion product of ingested β-β carotene (via isocryptoxanthin) and that this conversion occurred in the ovary and not in the gut wall. Tsushima et al. (1993) subsequently showed β-carotene to be the precursor for echinenone, via β-isocryptoxanthin, in *Pseudocentrotus depressus*. However, Goebel and Barker (1998) have shown that feeding processed carrot pulp or synthetic β-carotene supplements to *Evechinus chloroticus* produced no significant effect on the gonad color.

Gonad growth was shown to be significantly enhanced in *Psammechinus miliaris* when fed on commercially prepared salmon diets, either directly or in polycultured systems (Kelly et al. 1998b; Cook et al. 1998). Although the salmon feed contained the carotenoid astaxanthin, these urchins produced gonads of a duller orange than the bright and most desirable shades found occasionally in local wild populations of *P. miliaris*. Similarly, Havardsson and Imsland (1999) found no evidence to suggest *S. droebachiensis* could utilize such higher oxidation state pigments as astaxanthin as metabolic precursors for echinenone.

Wild *P. miliaris* are often found feeding on the macroalgae *Laminaria saccharina* (Kelly 2000) and a range of encrusting invertebrate species (Lawrence 1975). The addition of macroalgae as a source of carotenoids in urchin artificial diets may enhance gonad color; however, macroalgae typically exhibit seasonal biochemical variations (Black 1950) that could result in variability of the final product. However, microalgae that contain carotenoids can be cultured under controlled conditions and on a large scale using existing technology. The aim of this investigation, therefore, was to determine if incorporating carotenoid-rich microalgae into artificial diets for *P. miliaris* had a positive effect on gonad color and growth.

MATERIALS AND METHODS

Sea Urchin Collection and Maintenance

Psammechinus miliaris were collected by SCUBA from a depth of 5 m in Loch Creran, Scotland (56°32′20″N; 5°17′00″W).

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Twenty-two urchins (horizontal test diameter 20–30 mm), selected at random, were placed into each of 12 10:1 aquaria. Each aquarium had an independent supply of 250 μm filtered seawater at ambient temperature and salinity. The experiment was conducted over twelve weeks from May to August 1999, when the seawater temperature ranged from 9.9–15.3°C. The photoperiod was maintained at a constant 16-h light: 8-h dark cycle throughout the experiment.

The urchins were left to acclimatize to the aquarium conditions for a period of sixteen days before the start of the experiment, during which time they were starved to standardize their nutritional status (Vadas 1977). The urchins were then fed one of four diets at a constant rate of approximately 3% of their mean body weight per day. Any waste food and feces were removed every second day by carefully siphoning around the urchins.

Diet Preparation

The diets were allocated to aquaria using a randomized block design, with three replicates of each treatment. The diets were made from commercially available raw materials (Table 1) with either additional micro, macro, or no additional algae (as a control). Agar and gelatine were chosen as suitable binding agents (Caltagirone et al. 1992), and the food pellets retained a firm consistency in seawater when subjected to urchin grazing. The microalgae were obtained from the Culture Collection of Algae and Protozoa (CCAP), Dunstaffnage Marine Lab., Oban, UK. The species used were the diatom *Phaeodactylum tricornutum* Bohlin (CCAP 19/18) and the flagellate Tahitian *Isochrysis* sp. (Parke) (T-ISO, CCAP 927/14). The macroalgae *Laminaria saccharina* (L) Lamour was collected locally.

The microalgal strains were selected on the basis of their pigment profiles and their growth characteristics. The diatom *P. tricornutum*, belonging to the Bacillariophyceae, has as its major carotenoid pigments the xanthophylls fucoxanthin and diadinoxanthin and small amounts (<1% of total carotenoid content) of β-β carotene and cis-β-β carotene (Wright et al. 1991). T-ISO, a golden-brown flagellate (Prymnesiophyceae) similarly contains the carotenoids fucoxanthin, diadinoxanthin, and smaller amounts of β-β carotene (Brown et al. 1993). *L. saccharina* has fucoxanthin as its primary xanthophyll and violaxanthin as the second major xanthophyll. β-β carotene is the only carotene present, zeaxanthin, neoxanthin, and fucoxanthinol are found at the 0.01–0.02% of total carotenoid level (Haugan & Liaaenjensen 1994).

The microalgal cultures were grown in a semicontinuous batch cultivation system, maintained at 20°C (± 2°C) and constantly illuminated and aerated. The growth medium was autoclaved seawater enriched with 1 mL L^{-1} Walnes medium and 0.1 mL L^{-1} vitamin solution. In addition, 0.3 mL L^{-1} of sodium metasilicate (Na_2SiO_3 0.5H2O) was added to the *P. tricornutum* cultures. Before harvest, the cell densities were calculated from haemocytometer counts; cells were harvested when the densities were approximately 1×10^7 cells ml l^{-1}. The algae were separated from their culture media using centrifugation (8,000 rpm, 10 min). Approximately 6–9 L of T-ISO and 3–4 L of *P. tricornutum* cultures were required to yield a soft algal pellet of 4–6 g wet weight. The *L. saccharina* was rinsed in filtered seawater and finely chopped before incorporation into the diets. The dry ingredients and the corn oil were weighed into a beaker, the appropriate amount of water added, and the mixture thoroughly stirred. The mixture was then heated in the microwave for 15 sec, stirred briefly, and then heated for a further 15 sec. It was then allowed to cool to approximately 35 C before adding the algae. Once set, the prepared diets were cut into 1-cm^2 cubes before distribution to the urchins at a rate of one cube $individual^{-1}$ day^{-1}.

TABLE 1.

Diet composition.

Ingredient	Amount	Percentage Wet Weight
Corn oil	0.12 g	7.18%
Dried skimmed milk	0.63 g	4.64%
Casein	0.57 g	7.95%
Gelatine	0.37 g	6.72%
Agar	0.53 g	1.57%
Filtered seawater	25mL/20mL	
Algae	4.5–5g	
	(0 g for control diet)	70.84%

Data Collection and Statistical Analysis

Five urchins from each aquarium were sacrificed at the start of the experimental period (sample day 1, 20 May, 1999) and thereafter at approximately monthly intervals. Whole wet weight (to the nearest 0.001 g) and horizontal test diameter (to the nearest 0.05 mm) were recorded before dissection. Upon dissection, the color of the gonads was assessed immediately by matching it to the closest color in the Pantone® collection of standards (Pantone 1995, Cook 1999). The gonad color was always assessed by the same observer in good natural daylight. Unmarketable shades were dark brown, gray, or black (Pantone colors 4–6, 1545, and 161); acceptable colors were pale yellow and orange (Pantone 155, 156, 713), and the most desirable shades were bright orange (Pantone 123, 136, 149).

The wet weight of the gonad, emptied gut, and eviscerated test were also recorded. Whenever possible, the sex of the individual was determined by examination of extruded gametes, because sex can influence gonad color, ripe males tending to paler colors. The gonad index (GI) was calculated as a measure of gonad growth. An alimentary index (AI) was calculated as an additional indicator of sequestered nutrient stores (Klinger et al. 1998, Kelly et al., 1998b). Indices were calculated using the formulae of Kelly et al., 1998a: GI (or AI) = wet weight of gonads (or gut) divided by the wet weight of the eviscerated test and spines, expressed as a percentage. To allow comparison with GI calculated using total wet weight as the denominator, the GI values quoted here should be divided by a conversion factor of 1.45.

The numerical data were log-transformed and tested for normality and homogeneity of variance (Zar 1996) using MINITAB, version 12.1 for Windows. The log-transformed data were analyzed using analysis of variance (ANOVA) when the assumptions were met, and the Kruskal–Wallis nonparametric ANOVA when violations occurred. Tukey's or the nonparametric Nemenyis (Zar 1996) multiple comparisons tests were employed to assess where the differences occurred in the cases of rejection of the null hypothesis. The observed gonad colors were classified as unacceptable, acceptable, or excellent for marketing. The frequencies of occurrence in each category at each sample date were compared to those expected should the algae have had no effect on color (i.e., those observed in the control group) using the log-likelihood ratio (Zar 1996).

RESULTS

Gonad Growth

A three-way ANOVA incorporating sample date and treatment as fixed factors and replicate tanks nested within treatments showed that there was a significant increase in GI over the duration of the experiment ($F = 64.67$, $df = 2, 16$, $P < 0.001$) (Fig. 1).

Nested (replicate tanks within treatments) ANOVA was used to examine difference in GI between treatments on any given sample day. At the start of the experiment (sample 1), there were no significant differences in gonad index between diet groups. At the end of the experiment, both groups of microalgae-fed (*P. tricornutum* and T-ISO) urchins had significantly higher ($F = 4.0$, $P = 0.013$, $df = 3$) GIs than those fed the control diet. There was no significant difference in GI between urchins fed *L. saccharina* and the control group. The GI produced by *P. tricornutum* became significantly higher than that produced by the control diet after only five weeks; whereas, the effect of T-ISO did not become significant until the end of the trial (after twelve weeks).

Alimentary Indices

There were no significant treatment effects on AI at any time during the experiment (sample 1: $P = 0.754$, sample 2: $P = 0.097$, sample 3: $P = 0.536$, sample 4: $P = 0.424$). There was, however, a significant increase in the AI of all the algae-fed urchins after the first five weeks of the trial (*L. saccharina*-fed urchins: $P < 0.001$, *P. tricornutum*-fed urchins: $P < 0.001$ and T-ISO-fed urchins: $P < 0.001$). The urchins fed the control diet did not show a significant increase in AI until eight weeks into the trial ($P < 0.001$). There was no significant change in test diameter over the duration of the trial ($P = 0.198$) or among diets on individual sample dates ($P = 0.844$).

Gonad Color and Sex Distribution

The gonad color distribution for each treatment at each sample date is summarized in Table 2. At the start of the experiment, there were no differences in color distribution between any of the treatment groups and the control group. However, after five weeks (sample 2), the urchins fed *P. tricornutum* had a significantly better color distribution than those fed the control diet (Table 3). After twelve weeks, the T-ISO fed urchins also had a significantly better color distribution than the control group. The data generated from the control group indicated there was no seasonal change in gonad color over the course of the experiment.

The sex ratios on sample days three and four were determined as the urchins spawned on dissection, and a log-likelihood comparison with the ratio in the control group showed that the male:female ratio for urchins fed the T-ISO diet differed significantly from that of the control on sample days 3 and 4 (Table 4).

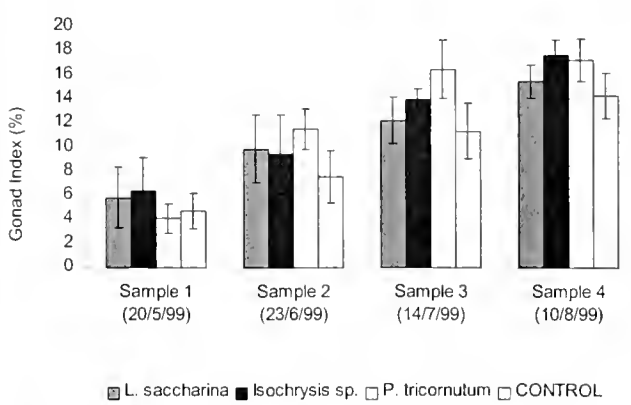

Figure 1. Mean gonad indices at each sample date, with 95% confidence intervals.

DISCUSSION

Gonadal and Somatic Growth

The gonad acts as a nutrient storage organ in *Psammechinus miliaris*; urchins provided with a nutritious food source demonstrate rapid gonad growth because of a proliferation of nutritive phagocytes (Kelly et al. 1998b). The low GIs of the urchins at the start of this experiment suggest that the wild population at the collection site were of a poor nutritional status. Therefore, it is not surprising that the GI in all treatment groups increased significantly over the experimental period, during which they were receiving a constant supply of nutritious food. However, by the end of the experimental period, the GIs of the urchins fed the two microalgal diets were significantly higher than those fed the control diet, implying that the microalgae imparted additional nutritional value for the urchins.

The gonad biomass of many commercially important echinoids shows a strong seasonal variation related to their annual reproductive cycle, resulting in a limited season of economically viable harvest (Byrne 1990, Hagen 1998). This experiment was conducted during the spawning season of *P. miliaris* in Scotland (Kelly 2000) during which gamete release normally results in a steady decline in GI. However, instead of decreasing, the GI increased steadily over the duration of the experiment, indicating it is possible to have an out-of-season gonad yield from *P. miliaris* fed artificial diets enhanced with microalgae.

Despite an approximate threefold increase during the course of the experiment, the mean values of GI obtained for all groups by the end of the trial (14.25–17.64%, $n = 15$) remained lower than the maximum values obtained in other studies on this species in Scotland. Kelly et al. (1998b) found that urchins reared in proximity to Atlantic salmon (*Salmo salar*) in polyculture trials could attain summer GIs of as much as 40%. In laboratory trials, Cook et al. (1998) found that *P. miliaris* fed commercially manufactured salmon food attained a mean GI of approximately 35% in August, which continued to grow for the remainder of the trial, reaching a maximum value of almost 60% by December. The lower values obtained in this trial are likely to be a result of the inferior nutritional value of the microalgal diets, as compared to commercially manufactured salmon food. Although a full comparison of the relative nutritional profile of the algae used was beyond the scope of this study, it is interesting to note that *Phaeodactylum tricornutum* and *Isocrysis* sp. are reported to have a high content of the fatty acid EPA (Otero et al. 1997) and DHA (Fabregas et al. 1995), which may partially account for the enhanced gonad growth of urchins fed these diets. Enhanced gonad growth has been demonstrated for *P. miliaris* fed diets rich in DHA (Cook et al. 2000).

The AI is an additional measure of the nutritional status of echinoids, because it is also a site of nutrient storage (Lawrence et al. 1966, Lawrence & Lane 1982). In this trial, the gut indices of all groups increased significantly during the first five weeks of the trial, further implying that the wild urchins had a low nutritional status at the start of the trial. However, there was no further in-

TABLE 2.

Contingency table summarizing the observed frequencies of gonad color categories.

	Sample 1 (20th May 1999)			Sample 2 (23rd June 1999)			Sample 3 (14th July 1999)			Sample 4 (10th August 1999)		
	u	a	e	u	a	e	u	a	e	u	a	e
Control	60	20	20	80	7	13	47	40	13	53	40	7
L. saccharina	80	13	7	53	13	33	47	40	13	47	40	13
T-ISO	73	7	20	60	7	33	40	40	20	20	40	40
P. tricornutum	86	7	7	40	0	60	33	7	60	7	40	53

(u = unacceptable; a = acceptable and e = excellent) $n = 15$.

crease in AI over the remainder of the experiment, reflecting either the limited storage capacity of the gut wall (Lawrence & Lane 1982) or the limiting nutritional value of the experimental diets. No significant somatic (test) growth was observed over the duration of the experiment. However Cook et al. (1998) found that adult urchins fed a macroalgal diet increased in test diameter at an average rate of only 0.02 mm/month.

Gonad Color

Relatively few studies have been designed to address the important issue of gonad color in sea urchin production specifically. The measurement and communication of color requires careful attention, and although several studies (Barker et al. 1998, Watts et al. 1998) have noted that differences in gonad color occur in echinoids fed artificial diets, they do not attempt to quantify their comparisons. The use of internationally recognized color standards in the present trial was intended to provide a less ambiguous means of color communication and comparison.

The use of synthetic carotenoid pigments to improve flesh coloration is common in the manufacture of artificial diets for cultured salmonid fish (Storebakken et al. 1987, Foss et al. 1987). However, the cost of synthetic pigments contributes significantly to the production cost of artificial feeds in salmonid aquaculture (Torrissen et al. 1990); therefore, it should be avoided if possible in echinoculture.

Robinson and Castell (2000) have shown that incorporating a spray-dried form of the microalgae *Dunaliella salina*, which is rich in β-carotene, into artificial diets caused a pronounced improvement in gonad color in *Strongylocentrotus droebachiensis*. Goebel and Barker (1998) noted no improvement in the gonad color of *E. chloroticus* when fed β-carotene supplements. This suggests that the form in which the pigment is supplied in the diet also dictates how or if it is later expressed in the gonad. Obviously, it would be of interest to compare the effect of a range of different pigments on the gonad color of a variety of commercially important echinoid species. A detailed comparison of the pigment profiles of urchin gonads of a "good" and "poor" color for each species may also help elucidate the pigment requirements of effective, color-enhancing artificial diets. Pigment expression in the gonad may be linked to factors other than pigment presence alone; for example, lipid content or the presence of gametes.

In the spawning season, the ovaries contain more pigment than the testes (Goodwin 1984). Griffiths and Perrott (1976) have shown that carotenoids are passed from the ovaries to the eggs, and Hallenstvet et al. (1978) subsequently showed that they are then transferred to the larvae. It is thought that these pigments play an important protective role in larval development and biological defense (Kawakami et al. 1998). Consideration of sex ratios is, therefore, important in color comparisons between treatment groups. In this study, it was noted that, in general, the female urchins produced gonads of more marketable colors than males, presumably because of the influence of the pigmented eggs as opposed to sperm. At both sample dates three and four, the sex ratios were effectively the same in the control group and the *P. tricornutum*-fed group, the two groups between which the biggest differences in color were seen. This indicates that the observed improvement in gonad color was not merely attributable to unequal sex ratios in the samples, but was a true effect of the diet. In the T-ISO-fed group; however, there were more males than females at sample day three and then more females than males at sample day four. Because the significant shift in color distribution for T-ISO-fed urchins also occurred between these two dates, this result should perhaps be interpreted with a greater degree of caution.

The results of this trial indicate that the microalga *P. tricornu-*

TABLE 3.

Comparison of gonad color between each treatment group and the control group at the four sample dates of the experiment.

	Sample 1 (20th May 1999)*	Sample 2 (23rd June 1999)	Sample 3 (14th July 1999)	Sample 4 (10th August 1999)
L. saccharina	G = 3.085	G = 5.448	G = 0	G = 0.903
T-ISO	G = 2.218	G = 3.985	G = 0.583	G = 15.616*
P. tricornutum	G = 5.166	G = 18.756*	G = 20.125*	G = 29.112*

* Indicates a distribution having a significantly different unacceptable: acceptable: excellent ratio from the control group at the given time. $G_{critical} = \chi^2_{0.05,2} = 5.991$ (Zar 1996).

TABLE 4.

Comparisons of male:female ratios between treatment groups in the trial (after eight and twelve weeks).

	T-ISO	P. tricornutum	L. saccharina	Control
Sample 3	10:5	4:11	8:7	6:9
(14 July '99)	(G = 4.339)*	(G = 1.171)	(G = 1.085)	
Sample 4	6:9	9:6	8:7	10:5
(10 August '99)	(G = 4.450)*	(G = 0.291)	(G = 1.140)	

* Indicates the male:female ratio is significantly different from that of the control group at the given date.
$G_{critical} = \chi^2_{0.05,1} = 3.841$ (Zar 1996).

tum is a better choice for inclusion in artificial diets for sea urchins that the other algae considered. Not only did it result in significantly better gonad growth; it also significantly improved gonad color distribution. Furthermore, the improvement in color occurred in a much shorter time, having important implications for culture if microalgal diets are to be used as preharvest conditioning diets for wild or polycultured urchins. In addition, *P. tricornutum* is exceptionally robust in culture and performs well in semicontinuous culture (Otero et al. 1997). It is not clear at present whether the weakly silicified cell walls *P. tricornutum* (Bold & Wynne 1985) affects the ability of the urchins to assimilate the pigment from the algae or pigment preservation during diet manufacture. Although reported to contain the same carotenoids as *P. tricornutum*, the *L. saccharina* diet did not improve gonad color in comparison with the control. Accurate pigment profiles of the algal cultures actually used to create experimental diets would benefit artificial diet design, because the biochemical and pigment composition of algae can change with nutrient and light availability (Fabregas et al. 1995, Fabregas et al. 1998) and season (Stengel & Dring 1998).

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TECHNICAL PAPERS

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LITTORINE FORAGING BEHAVIOR AND POPULATION STRUCTURE ON A WAVE-EXPOSED SHORE: NON-LINEAR RESPONSES ACROSS A PHYSICAL GRADIENT

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ABSTRACT Littorines often use topographical features (e.g., holes, crevices) for shelter. We investigated this behavior (% outside of natural crevices and artificial holes during daytime low tides) over a cross-shore wave force gradient. Whereas maximal wave force (MWF) declined linearly with increasing distance from the low tide mark, percent outside increased along this gradient and reached an asymptotic level (70%) 40 m from the low tide mark. A comparison of seasonal averages of percent outside and MWF showed an abrupt transition between high and low values of percent outside with increasing MWF, but comparisons of daily data were more varied, presumably due to the superimposition of other environmental factors (e.g., desiccation). Contrary to earlier descriptive studies and biomechanical predictions, larger littorines were found in locations closer to the low tide mark where MWF were higher, suggesting that size-limitation from hydrodynamic forces does not occur within the size range studied (1–8 mm) when shelters are available. However, the restriction of littorines to areas near shelters can greatly influence community structure by permitting the development of algal assemblages in exposed locations.

KEY WORDS: gastropods, grazing, heterogeneity, hydrodynamic forces, intertidal ecology, refuges, and size gradients

INTRODUCTION

Ecologists have often examined species over environmental gradients in which specific predictions can be made regarding the responses of the species under study. Recent examples include grazing responses of arctic grasses across a snow deposition gradient (Wegener & Odasz 1997) and predation on a marine snail across an algal gradient (Alfaro & Carpenter 1999). Although this approach can only provide correlative relationships, it is especially useful for examining factors that are difficult to manipulate experimentally.

Striking physical gradients occur in intertidal environments, and the distribution of intertidal organisms (e.g., zonation patterns) is often thought to be a function of physical gradients acting either directly on them or indirectly on their predators or competitors (e.g., Garrity 1984, Takada 1996). One well recognized gradient is "wave exposure," which has been typically examined at sites along the shoreline, e.g., from a headland into a bay (Lewis 1964, Palumbi 1986). Intuitively, a gradient in wave energy should also exist across the shoreline as breaking waves dissipate their energy, but this gradient has never been quantified in rocky intertidal environments.

The biology of intertidal organisms has often been examined with respect to this physical gradient (e.g., Emson & Faller-Fristch 1976, Atkinson & Newbury 1984, Chapman 1994, Britton 1995, Takada 1996). Unfortunately, many studies have only examined the environment at two locations along the gradient, often at the extremes, and then interpolated for intermediate locations. Moreover, wave exposure has rarely been quantified and has usually just been subjectively described as "exposed" or "protected." While the most important aspect of water motion is sometimes unclear for a particular situation (Denny et al. 1985, Denny 1988), a number of investigators have developed techniques for measuring maximum wave forces (Jones & Demetropoulus 1968, Palumbi 1984, Bell & Denny 1994), the parameter of water motion most related to dislodgment.

The risk of dislodgment is thought to influence the biology of littorine snails, the dominant herbivores of many high- and mid-shore rocky intertidal habitats, and both morphological and behavioral traits of littorines have been interpreted as responses to water motion. These include larger relative foot area (Atkinson & Newbury 1984, Trussell et al. 1993), shell morphology (Johannesson 1986, Trussell et al. 1993, Trussell 1997, Boulding et al. 1999), and the use of shelters (Emson & Faller-Fritsch 1976, Raffaelli & Hughes 1978, Atkinson & Newbury 1984, Chapman 1994). This latter behavioral aspect can be quite important in influencing both the density and size frequence of littorinid populations (Emson & Faller-Fritsch 1976, Raffaelli & Hughes 1978, Atkinson & Newbury 1984, Trussell et al. 1993). While the influence of crevices on both the individual behavior and population structure of littorinids has been explored, there has been a lack of attention to the physical environment that allegedly controls the behavior of these snails. Although past studies may have been qualitatively correct in their estimation of "wave exposure," there remains a need to explore quantitatively both the behavior and the physical environment to develop a predictive understanding of the ecology of littorines and intertidal communities.

In this study, measures of the physical environment are combined with ecological data to explore the control of foraging behavior across an environmental gradient. Specifically, we examine the correlative relationship between maximum wave force and the use of crevices across an intertidal shore. Such behavioral studies of intertidal invertebrates are rare, as active behavior usually occurs when the tide is high and researchers are absent. Littorines offer an exceptional opportunity as they are often active during low tide (Jones & Boulding 1999) and are distributed in the mid- and high intertidal zones, thereby permitting prolonged periods for observations.

MATERIALS AND METHODS

The study was conducted at Pointe Métis (48°41′N and 68°02′W) located on the St. Lawrence maritime estuary near Mont Joli in Quebec, Canada. Two sites were used: one consisted of a series of bedrock ridges paralleling the shoreline with consolidated boulders and cobbles between the ridges (Fig. 1; Site 1), the other

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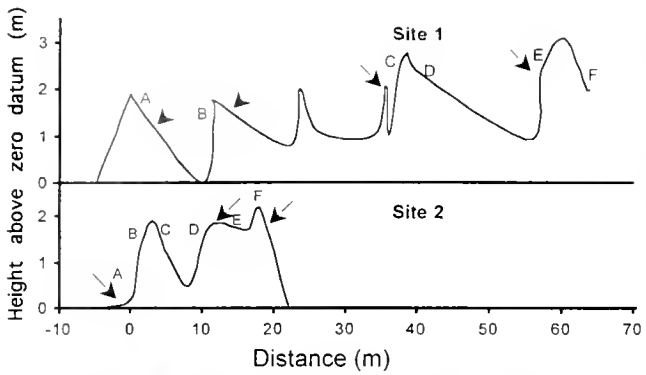

Figure 1. Cross-shore profiles of study sites (note exaggerated vertical scale). All measurements are based on the 0 m low tide mark established for each site. Letters represent the locations censused for littorine behavior. Arrows indicate the locations of maximal wave force transducers.

was a series of less well-defined ridges with intervening tide pools (Fig. 1; Site 2). Elevated rock surfaces at both sites supported few sessile organisms (algae or invertebrates) due to the annual ice scour that occurs along the shores of the estuary (Archambault & Bourget 1983). Littorine snails, principally *Littorina saxatilis* Olivi, but on occasion *L. obtusata* L., were the most conspicuous animals in this habitat and often aggregated in crevices and holes when not foraging on the open rock surface.

The behavior of littorines was studied along a transect perpendicular to shore at each site. At Site 1, the transect covered approximately 70 m and ran across five distinct ridges. At Site 2, the transect covered approximately 20 m and ran across four ridges. Six specific locations were selected along each transect for observations, each corresponding to a natural feature of the landscape (e.g., a face of a ridge). For the three most seaward locations at Site 1, 30-cm portions of six crevices spaced 0.5–2 m apart were chosen at each location (18 total). At Site 2, three crevices were sampled at the most seaward site and at two mid-transect locations (nine total). For each location, tidal height, distance from the spring low tide mark, orientation and inclination of the rock face were measured as well as the dimensions (average of six measures of width and depth) of each crevice (Table 1). Littorines present within the crevices ("inside") as well as the littorines that were present on the exposed rock surface within 25 cm of the crevices ("outside") were counted, and percent outside calculated as 100×(number outside)/(number inside and outside). The distance of 25 cm was chosen based on earlier observations of browse zones (typically 10–20 cm in width) in the algal mats found at certain locations along the transects (L.E. Johnson, pers. obs.). When other shelters were found within 50 cm of the crevice, only littorines within half the distance between the crevice and the other shelter were counted. Local density was calculated as the number (inside + outside) per cm^3 of crevice volume, calculated assuming a triangular cross-section (i.e.,volume = 0.5 × width × depth × length).

In addition to these naturally occurring but variable irregularities in the rock surface, we created standardized irregularities in early May 1998 by drilling sets of holes at all locations along the transects to control for any variation in the form of the shelters across this shoreline. Again, tidal height, distance, orientation and inclination of the rock face were recorded (Table 1). Each set of holes consisted of three holes 1.3 cm in width and 1 cm in depth (total volume = 4.0 cm^3). Each of the holes within the set was

TABLE 1.

Descriptive information on natural crevices and artificial holes used at sites 1 and 2 for examining littorine behavior.

Holes:	Site 1					Site 2				
Location	Dist. (m)	Height (m)	Face	Inclin.	Density (no/cm^3)	Dist. (m)	Height (m)	Face	Inclin.	Density (no/cm^3)
A	0.1	1.8	South	35	5.4^{c}	0.3	0.2	hori.	0	3.3^{cd}
B	11.7	1.6	North	90	7.6^{c}	1.0	0.7	North	90	5.0^{bc}
C	37.6	2.4	North	50	18.4^{a}	3.0	1.2	South	65	3.2^{cd}
D	39.8	2.4	South	70	2.5^{d}	11.5	0.2	West	40	10.5^{a}
E	57.2	2.3	North	90	16.4^{ab}	16.1	1.8	North	10	10.6^{a}
F	63.6	2.0	South	35	13.0^{b}	17.0	2.2	hori.	0	7.6^{b}
Crevice: Site 1										
Location	Dist. (m)	Height (m)	Face	Inclin.	Depth (mm)	Width (mm)	Vol. (cm^3)	Density (no/cm^3)		
A	0.1	1.8	South	35	16.1	17.2	41.5	2.1^{d}		
B	11.7	1.8	North	90	15.2	14.6	33.3	2.1^{d}		
C	37.6	2.4	North	50	15.9	14.1	33.6	2.9^{d}		
Crevice: Site 2										
Location	Dist. (m)	Height (m)	Face	Inclin.	Depth (mm)	Width (mm)	Vol. (cm^3)	Density (no/cm^3)		
A	1.0	0.2	hori.	0	15.6	10.6	24.8	1.7^{d}		
D	11.5	1.7	West	40	17.9	19.3	51.8	2.6^{cd}		
E	16.0	1.8	North	10	12.5	12.1	22.7	4.0^{cd}		

Descriptive information on natural crevices and artificial holes used at sites 1 and 2 for examining littorine behavior: distance from the low tide mark, tidal height, orientation and inclination from the horizontal of the rock face, as well as crevice depth, width, volume (sets of holes always had a total volume of 4.0 cm^3), and densities (no./cm^3 shelter) for each location.

$^{a-d}$ For densities, similar letters indicate no significant differences between groups for multiple pair-wise analyses of two-factor ANOVA (Table 2) performed for each site separately.

separated by 5 cm in a triangular pattern. Three replicates of these sets of holes were established at each location (except one where six were established as a pilot study of the influence of adjacent algal abundance). For each period of observation, the littorines were enumerated as above, except a distance of 15 cm from the holes was used as holes were usually on rock surfaces with few other irregularities and most littorines were found within this distance.

Littorines were counted daily during the morning low tide for series of 3–7 days during spring tides on an approximately biweekly schedule from May 29 to September 20 in 1998 at both sites and May 30 to August 30 in 1999 at Site 1 only, for a total of 64 days of observations. Limited observations were also made during other periods of the spring-neap tide cycle and during other seasons. Littorines were also visually classified with minimal disturbance into 4 different size categories: 1–2 mm, 2-4 mm, 4–6 mm, and 6+ mm (smaller littorines, < 1 mm, were difficult to discriminate against the rock surface). While littorines are usually sized by shell length, we used shell height as defined by Fletcher (1995) as "the distance of the highest point of the shell above a flat surface upon which the shell is lying with the aperture facing downwards" as this measure appears more relevant to the use of crevices.

To estimate the wave energy gradient along these transects, maximum wave force (MWF) was measured over 24 hr simultaneously at three or four locations that corresponded approximately to a subset of the locations of the shelters (Fig. 1). In 1998, only one measurement was taken at each location (Site 1: locations A, B, C, and E; Site 2: locations A, D, and F) while in 1999 the number was increased to four (Site 1 only) to characterize better the MWF at a given location. Maximum wave force was measured using a simple transducer (Bell & Denny 1994) that consisted of a spring attached to a practice golf ball by monofilament line on which a rubber ring was able to slide. Only forces greater than 2 N were measurable by these transducers. The spring and ring were encased in PVC tubing and attached to the rock using a swivel attached to a screw anchored on a local high point. MWF was measured one day after devices were installed or reset. Such measurements were made simultaneously with behavioral observations on 18 days in 1998 and 23 days in 1999. Data from both sites in both years were used for seasonal comparisons of MWF and littorine behavior, whereas only data from Site 1 in 1999 were used to make daily comparisons. These measures of MWF were intended for relative comparisons among locations and not for estimating the forces experienced by the snails.

Data were analyzed using simple linear regressions or two-way ANOVAs with multiple pair-wise comparison tests (LS Means based on significant interactions when they occurred) for specific differences within significant sources of variation (SAS v. 6.12; SAS Institute Inc. 1996). When data were non-normal or variance unequal, we also analyzed rank-transformed data to confirm results obtained using non-transformed data.

RESULTS

Littorines were abundant the entire length of the transects but were 2 to 6 times denser in holes than in natural crevices based on the volume of the shelters available (Table 1). At Site 1, densities were higher at locations far from the low tide mark except at 40 m, a location with a steep, south-facing slope and a high number of crevices (Fig. 1 and Table 1). Densities along the shorter Site 2 transect were comparable to locations at similar distances at Site 1, but were also higher away from the low tide mark (Table 1). Densities in and around crevices were relatively constant (no significant differences) over the season and between years (Addy 2001). In contrast, after the holes were created in 1998, littorine densities in and around holes increased during the summer season but then stabilized in 1999 (Addy 2001). Littorine size structure varied similarly across both transects with larger (> 4 mm) individuals more abundant at locations near the low tide mark (Fig. 2). The percentage of littorines 2–4 mm remained relatively constant across the sites whereas that of the smallest littorines (1–2 mm) was more variable.

A lower percentage of littorines was found outside of holes and crevices at locations closer to the sea at both sites (Fig. 3). Percentage outside increased away from the low tide mark along both transects but became asymptotic near 40 m along the longer transect (Fig. 3a). This trend occurred for both natural crevices and artificial holes, although the percent outside was much higher around holes, typically double that of natural crevices. Although there were significant differences among different size classes in the percentages found outside of shelters at some specific locations, there were no obvious trends, and no statistical differences were found for pooled data, even when locations closer to the low water mark were analyzed separately from those farther away (Addy 2001).

Maximum wave force (MWF) decreased linearly with increasing distance from the low tide mark in both years, although maximum wave forces differed between the two years (Fig. 4). When seasonal means of MWF and percentage of littorines outside of holes are compared among locations, a nonlinear relationship is seen (Fig. 5) — the percentage outside of holes becomes exceed-

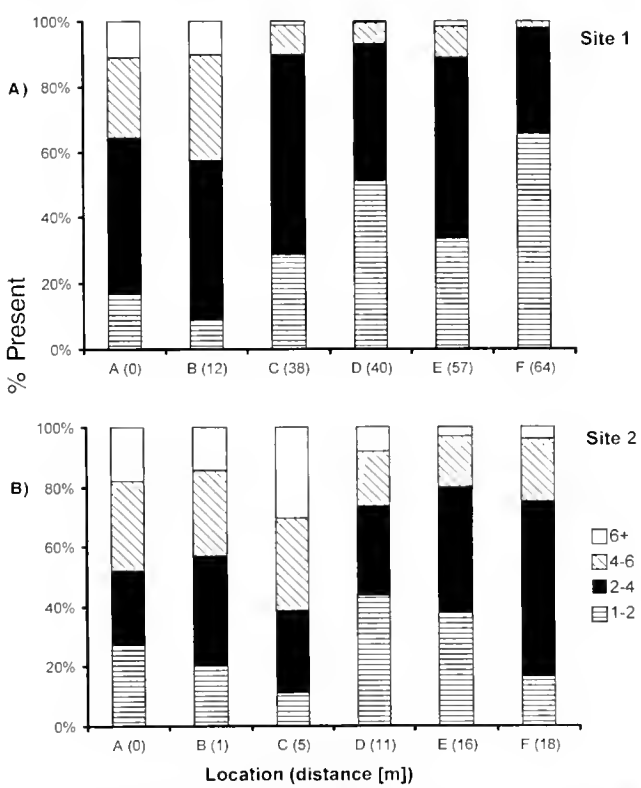

Figure 2. Percentage of each size class at locations of increasing distance from the low tide mark at (A) Site 1 and (B) Site 2.

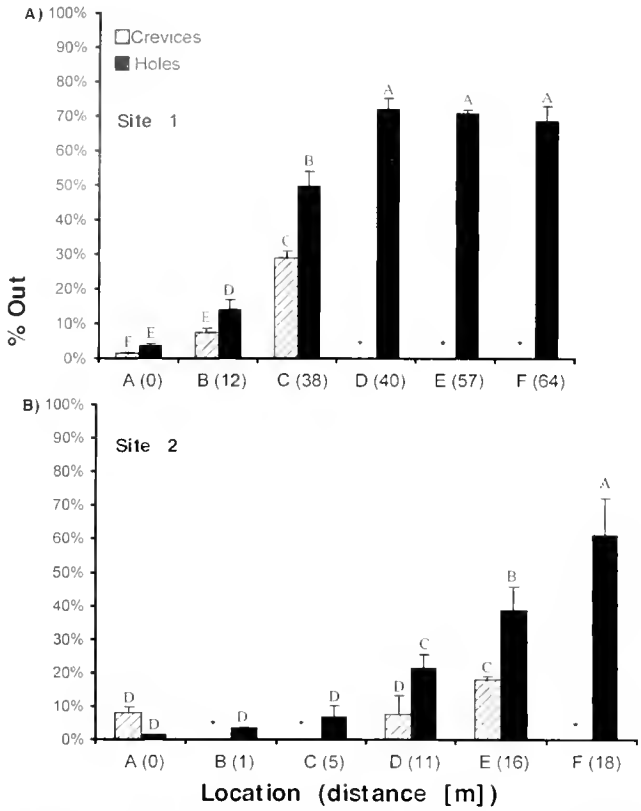

Figure 3. Percentage of littorines found outside of artificial holes and natural crevices at (A) Site 1 and (B) Site 2 for locations at increasing distance from the low tide mark. "*" indicates that crevice data were not collected for those locations. Similar letters indicate no significant differences between groups for multiple pair-wise analyses of two-factor ANOVA (Table 2) performed for each site separately.

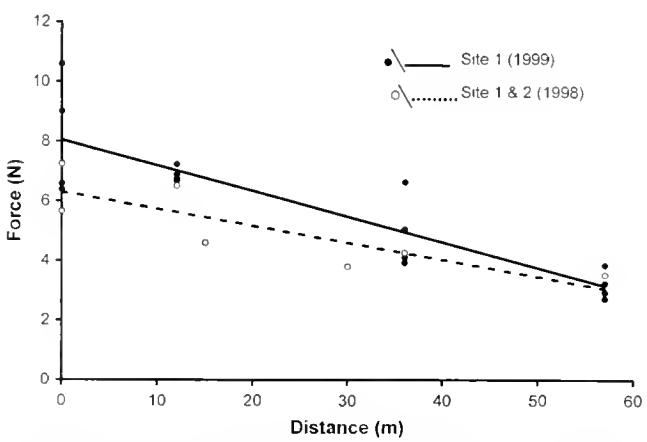

Figure 4. Average maximal wave forces (MWF) as a function of distance from low tide mark. Open circles and dashed line represent average MWF (22 d) for 7 locations (3 at Site 2 and 4 at Site 1) in summer of 1998 (n = 1 for each location); filled circles and solid line represent average MWF (23 d) recorded for four locations at Site 1 in summer of 1999 (n = 4 for each location). Lines represent linear regressions which are both significantly different from zero (1998: r^2 = 0.69, F = 11.4, $p < 0.02$; 1999: r^2 = 0.76, F = 44.5, $p < 0.001$).

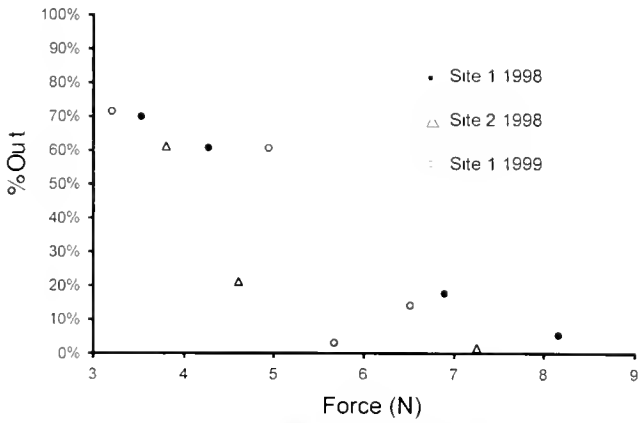

Figure 5. The relationship between the seasonal mean of the percentage of littorines found outside artificial holes and the seasonal mean of the maximal wave force (MWF). Each point represents a different location at Site 1 (1998 and 1999) or Site 2 (1998). Means for MWF in 1998 (n = 22 d) are from one MWF transducer at each location whereas means in 1999 (n = 23 d) are from four transducers at each location.

ingly low (mean < 15%) at locations where mean MWF exceeded a threshold of 5.0–5.5 N. Linear regressions of data above and below this obvious threshold were not significantly different from zero. The relationship between MWF and percent outside is less evident when data for each day are compared for each location (Fig. 6; data from four locations at Site 1 in 1999). Generally, locations closer to the low tide mark had lower values of percent outside, but the data were extremely variable at a given location for a given MWF (Fig. 6). Above a value of 8 N, the percentage outside dropped dramatically and remained near zero. The lowest values of percent outside for each location were obtained during a storm in which MWF exceeded 20 N at the most exposed location (Fig. 6).

DISCUSSION

In temperate environments, the use of crevices is generally thought to be a behavior for reducing the risk of dislodgment by

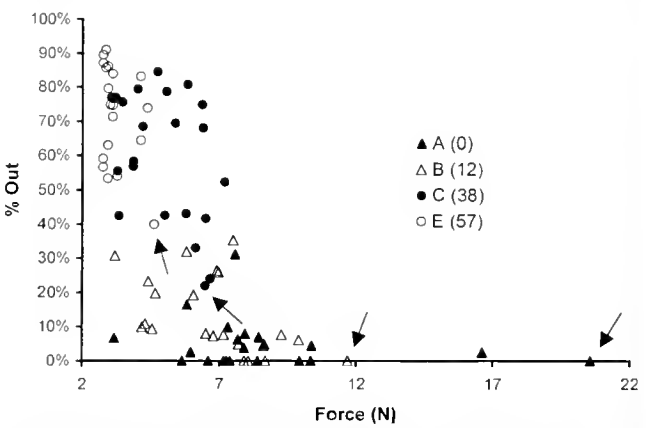

Figure 6. Comparisons of the percentage of littorines outside artificial holes versus maximal wave force (MWF) measured at four locations at Site 1 in 1999. Each point represents the average of three (percent outside) or four (MWF) measurements on a given day (n = 23 d). Values in parentheses on x-axis are horizontal distance from low tide mark. Arrows indicate data taken on the day after a storm.

waves (e.g., Emson & Faller-Fritsch 1976, Raffaelli & Hughes 1978). According to this idea, littorines limit their foraging activities to periods of low tide (Jones & Boulding 1999) and return to shelters to avoid being dislodged by the hydrodynamic forces of the incoming tide. Our results are consistent with this idea—the percentage of littorines foraging (i.e., outside of shelters) was lower in locations of higher maximal wave forces (MWF). An alternative explanation could be that littorines outside of shelters are dislodged at higher rates at locations of higher MWF, which would lead to a lower percentage of littorines outside of shelters but also to a lower local density unless recolonization occurred quickly. While we occasionally observed abrupt decreases in densities at some locations immediately following days of higher MWF, densities were in general quite constant over time (Addy 2001). Thus we interpret this relationship to be due more to behavioral responses (i.e., shelter-seeking behavior) than to demographic processes (i.e., "emigration" by dislodgment).

The relationship between wave force and shelter use is, however, strictly correlative and might be due to other factors. Density, for example, also generally increased with increasing distance from the low tide mark (Table 1). If densities reached a level where shelters were completely filled, then a higher percentage of littorines outside of shelters would occur simply due to a lack of available shelters. Although shelters were sometimes very full, this situation only occurred when values of percent outside were at or near zero. For over 90% of the observations at any given location, numbers of snails inside shelters were less than 75% of the maximum observed for that location (T.C. Addy, unpubl. data). In addition, locations with different densities had similar levels of percent outside (locations C and D at Site 1), and locations with similar densities had vastly different levels of percent outside (locations B and F at Site 2).

The linear decline in MWF with increasing distance from the low tide mark was not matched by a linear increase in the percentage of the littorines outside crevices. Instead, a plateau was reached at approximately 40 m, where MWF averaged 5–6 N, which may represent a threshold below which there is a reduced risk of dislodgment. When percent outside and wave forces were compared among individual days (Fig. 6), this general pattern was still apparent, but there was a great deal of variability in the percentage of littorines outside of shelters at any given location that was not explained by MWF. This variation appears to be related, at least in part to meteorological conditions experienced during low tide (e.g., temperature, desiccation). Other littorines reduce foraging during conditions of high evaporation (e.g. Atkinson & Newbury, 1984, Garrity 1984, Britton 1995), and in tropical environments, desiccation has been suggested as the leading reason for crevice-seeking behavior (Garrity 1984; but see Catesby & McKillup 1998 for the role of predation). We propose that desiccation plays a secondary role here as other field observations (day vs. night) and laboratory experiments (wet vs. dry rocks) support the idea that these littorines avoid conditions of high desiccation (Addy 2001).

Reductions in foraging time in locations of higher wave forces could ultimately have impacts on the population structure of littorine populations, i.e., smaller sizes and reduced densities. Our findings of larger littorines at locations near the low tide mark (Fig. 2) is contrary to this idea and the results of other studies where, if a size gradient existed, smaller littorines were more common close to the low tide mark (Vermeij 1972, Britton 1995 and references therein, Trussell et al. 1993). This trend has been attributed to the reduced susceptibility of smaller littorines to dislodgment (Trussell et al. 1993) and would be consistent with hydrodynamic theory (Denny et al. 1985). However, we examined relatively small littorines, and the trend could be different for larger sizes. Differences in crevice morphology among locations could also explain the trend we observed (e.g., if there were larger shelters in more exposed locations), but our use of artificial holes specifically controlled for this possibility. Thus the underlying cause of the differences in size distribution remains unknown and may be related to differences in local densities and concomitant competition for food. Regardless, the survival of larger littorines in more exposed locations may depend more on a shelter-seeking behavior that allows littorines to avoid hydrodynamic forces that might dislodge and displace them, thereby obscuring any relationship that might exist between size and the probability of dislodgment if shelters were not present. These littorines may be using crevices much like soldiers use foxholes when under fire.

An assessment of the relationship between wave force and littorine density is more difficult because the local abundances we report (i.e., number per unit volume of shelter) do not necessarily reflect the overall density of littorines at these locations or sites. This limitation notwithstanding, we saw increased local densities at locations farther from the low tide mark, which is consistent with the reduced probability of dislodgment there. Other factors (e.g., food supply, recruitment, densities of shelter) may also be acting. Likewise, marked differences in densities were observed over only several meters (Table 2: Location C vs. D, Site 1), suggesting again that other ecological processes are affecting densities.

Differences were observed between crevices and holes with regards to both local density and the percent outside of the shelters: holes sheltered more individuals for a given volume and those individuals appeared more likely to leave shelters during day-time low tides. Potential explanations for these differences are not obvious. The holes were a recent addition to the landscape, and thus were colonized by more actively moving individuals, which may also have been more likely to emerge during low tide. Regardless, the nature of the shelter (e.g., dimensions and location relative to other shelters) appears to have a strong influence on littorine foraging.

In this study, we have measured only a population-level response of littorines along a wave exposure gradient; thus an interpretation of individual responses is problematic. Without knowing the identity of individual snails, it is impossible to determine if the fraction of the population found outside of the shelter from day-to-day represents the same group of high risk-taking individuals, alternating subsets of the population, or a combination of the two possibilities. Still, the fact that some littorines stay inside shelter during periods when others leave is puzzling. This observation supports the idea that littorine behavior is likely to be a complex response to both environmental factors and physiological state (Jones & Boulding 1999), and we suggest that the risk of dislodgment interacts with more basic drives such as hunger or digestive processing in producing these patterns of behavior.

If the hydrodynamic environment is indeed controlling littorine behavior, it may also be indirectly affecting other elements of the intertidal community. At locations with high MWF, where the local densities of littorines and the percentage foraging are lower, green algal mats form when shelters are sufficiently sparse (i.e., more than approximately 30 cm apart). Although there may also be direct effects of water motion on these algae (e.g., wave splash reducing desiccation or higher turbulence increasing nutrient trans-

TABLE 2.

Results of two-way ANOVA on the local densities of littorines in and adjacent to shelters (crevices and holes) and percentage of littorines outside of shelters at locations of different distances from the low tide mark at the two sites.

Density	Site 1				Site 2			
Source	df	MS	F	p	df	MS	F	p
Model	8	150.7	30.4	<0.001	8	43.9	15.1	<0.001
Error	27	5.0			21	2.9		
Corr. totals	35				29			
Location	5	128.9	21.5	<0.001	5	33.1	11.4	<0.001
Shelter	1	390.7	78.7	<0.001	1	134.6	46.2	<0.001
Loc. × Shel.	2	85.4	17.2	<0.001	2	17.0	6.2	<0.01
% outside	Site 1				Site 2			
Source	df	MS	F	p	df	MS	F	p
Model	8	0.332	153	<0.001	8	0.117	13.1	<0.001
Error	27	0.002			21	0.009		
Corr. totals	35				29			
Location	5	0.324	149	<0.001	5	0.163	18.3	<0.001
Shelter	1	0.060	27.8	<0.001	1	0.041	4.5	<0.05
Loc. × Shel.	2	0.019	8.8	<0.01	2	0.032	3.6	<0.05

port), the reduced number of foraging littorines observed in areas of high wave action must contribute substantially to this phenomenon. However, additional quantitative documentation of the direct and indirect interactions of this community is needed to permit the development of a more predictive understanding of its nature.

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CONTRASTING LIFE HISTORIES AND DEMOGRAPHIES OF EIGHT SPECIES OF LITTORINES AT NINGALOO REEF, WESTERN AUSTRALIA

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ABSTRACT This study considers four species of littorines from rocky shores (*Nodilittorina australis*, *N. millegrana*, *N. trochoides*, and *Littoraria undulata*) and four species from mangroves (*L. cingulata*, *L. filosa*, *L. scabra*, and *L. sulculosa*) between July 1989 and June 1999. We conducted periodic censuses of the same replicate areas at four rocky shore sites spread over 40 km of shoreline and at the same replicate mangrove trees in four groups at sites at the opposite ends of a bay, about 2 km apart. This design allowed us to partition the variability in abundance of each species into components associated with the sites, sampling units within sites, time of sampling, sites × times of sampling, and the residual. The littorines of the rocky shores had the greatest variability associated with differences among the four sites for the total populations and among sites and sampling times × site for recruits, whereas the littorines of the mangroves showed the greatest variability associated with times of sampling and sites × times of sampling. We also conducted shorter-term mark and recapture studies to provide direct evidence about growth and survivorship of these snails. All three species of littorines from mangroves with sufficient recaptures showed rapid growth, reaching half their maximum size in 0.94, 0.35, and 0.75 y for *L. cingulata*, *L. filosa*, and *L. scabra*, respectively, and attaining maximum lengths of 22 to 27 mm. The littorines of the rocky shores all had slower growth than those from the mangroves and were all smaller in maximum size, from 10–17 mm. With the exception of *N. millegrana*, which took only 0.50 y to reach half maximum size, the snails on the rocky shores took much longer to reach half their maximal lengths than those in the mangroves (1.23, 2.87, and 1.28 y for *N. australis*, *N. trochoides*, and *L. undulata*, respectively). Patterns in survival parallel the estimates of times to reach half maximal size. We never recaptured *N. millegrana* over intervals as long as 1 y, but some *N. trochoides* marked in 1988 were still alive in 1995. This long-term study of eight similar species in a single geographic area highlights the variability in life histories and demographies of littorines.

KEY WORDS: *Nodilittorina*, *Littoraria*, spatial, temporal, abundance, growth, survival

INTRODUCTION

Littorine snails are such an abundant and conspicuous feature of upper intertidal marine habitats worldwide that they are the subjects of intensive and extensive taxonomic, ecological, evolutionary, physiological, and behavioral research (McQuaid 1996a, McQuaid 1996b). Nevertheless, ecological investigations of littorines, in common with almost all ecological studies, suffer from two shortcomings. First, most studies are of short duration, as pointed out for ecological studies in general by Tilman (1989). Although there are some exceptions involving short-lived species (e.g., Underwood & McFadyen 1983, Williams 1992), most population ecological studies do not last as long as the generation time of the littorines under investigation. Brown (1998) explained how the continuation of his studies on desert rodents revealed that interesting changes will always occur and need to be studied in long-term experiments. Second, there has been a geographic and associated taxonomic bias. Reflecting the majority of researchers, most studies have been in temperate Europe and North America dominated by the genus *Littorina*. Southern hemisphere and tropical littorines of the genera *Littoraria* and *Nodilittorina*, with important exceptions (e.g., Reid 1986), have not been as extensively and thoroughly studied (Davies & Williams 1998), and as pointed out by Underwood and Denley (1984), there are difficulties inherent in automatically applying the paradigms developed in one geographical area to another.

This long-term observational study of the population dynamics and life histories of littorines in tropical, northwestern Australia begins to address these issues of location and duration of study. By selecting Ningaloo Marine Park as a study site, we were able to study in one small area four species on mangroves and four from the rocky shore, of which three species are endemic to Western Australia. By returning to the same mangrove trees and sections of rocky shore at irregular intervals over a decade, we have been able to document spatial and temporal variability in abundances of each species over a time that exceeds the estimated maximum age of most littorines (Powell & Cummins 1985, Heller 1990). By performing simple mark and recapture studies, we have been able to estimate rates of important demographic processes. Thus, the objectives of this study were to (1) quantify the spatial and temporal variability in abundance of the eight species and of their recruitment and to examine the concordance of the temporal patterns in abundance, (2) estimate rates of growth and survival and so document the diversity of rates of these processes in relation to the changes in abundances, and (3) examine covariation in life-history characteristics.

MATERIALS AND METHODS

Study Sites

Our study sites were in the Ningaloo Marine Park in northwestern Western Australia (22°S, 114°E) (Fig. 1). We studied mangrove littorines in Mangrove Bay, a 2-km-wide bay with scattered *Avicennia marina* along its sandy shoreline. Except for a few trees at Yardie Creek, 40 km to the south, these mangroves are the last concentration of mangroves associated with the extensive stands in Exmouth Gulf to the east and are separated by 400 km from others at Carnarvon to the south. In fact, Reid (1986) indicated the absence of any species of mangrove littorine at Mangrove Bay, so their presence raises the question of the spatial and temporal variation in their distribution and abundance.

Rocky shores are more widespread, so we were able to study rocky-shore littorines at four locations spanning 22 km in Ningaloo Marine Park (Fig. 1). The rocky shores consist of horizontal

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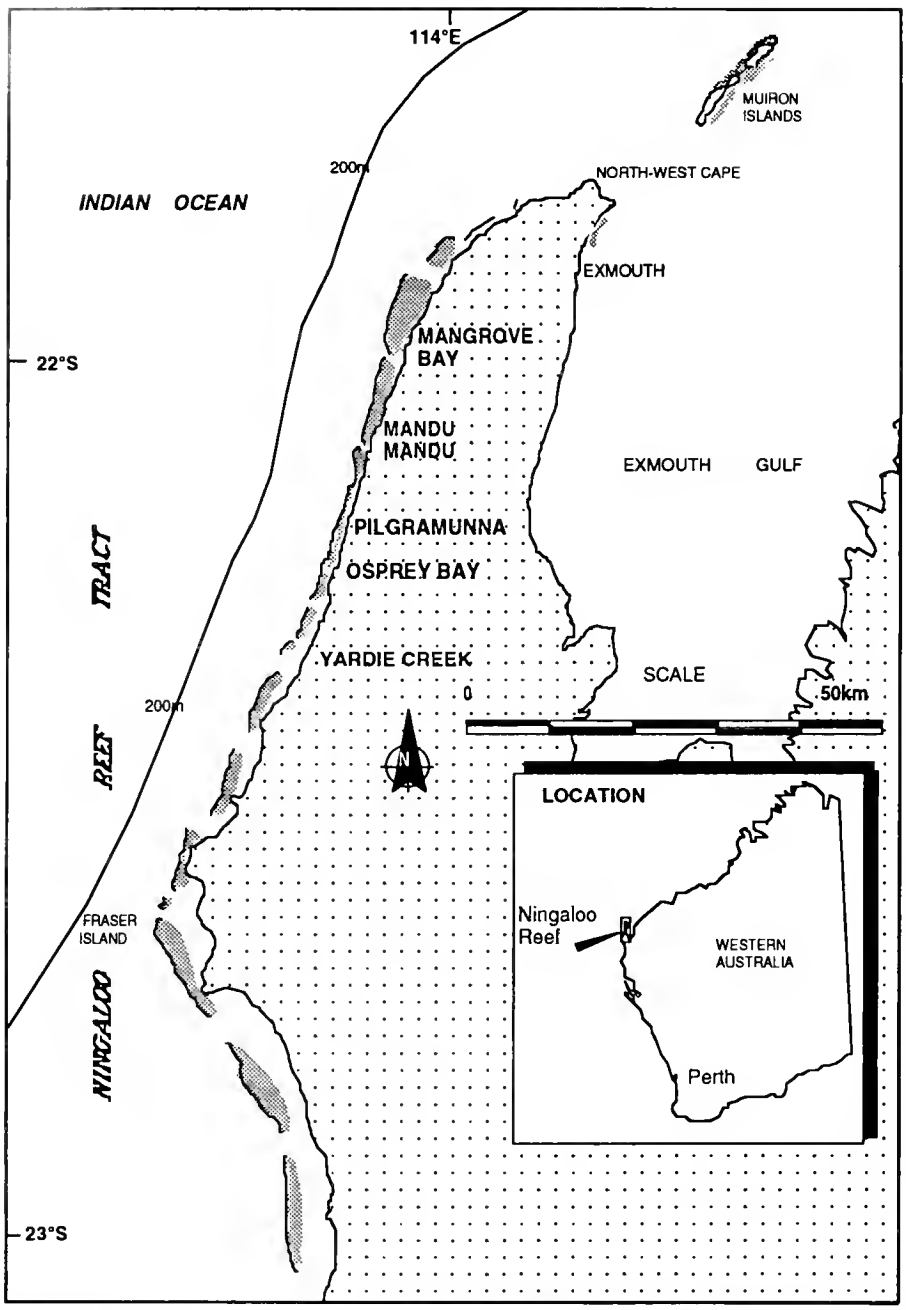

Figure 1. Map showing the five study sites on the mainland shoreline of Ningaloo Marine Park. We censused four species of mangrove littorines at Mangrove Bay and four species of rocky-shore littorines at Mandu Mandu, Pilgramunna, Osprey Bay, and Yardie Creek. The map also shows the discontinuous sections of fringing coral reef as a line for the reef crest, backed by the reef flat in stippling.

intertidal platforms that terminate in nearly vertical steps in the limestone up to 2.5 m high. The littorines live in the upper portion of the step and extend a small distance beyond the edge of the step. Each site consisted of several adjacent 1-m-wide vertical transects with a top horizontal portion that included the upper limit of distribution of the littorines and a larger vertical section that extended below the lower limit of distribution. The main site was at Mandu Mandu, which we chose because we had observed high densities of four species of littorines in 1987. The site was three adjacent 1-m-wide transects 9 m in length. The transects had a gently sloped upper section, a middle section of large blocks of rock, and a lower section of rock projecting from a cobble-covered, horizontal platform. The other three sites were south of Mandu Mandu at locations of easy access where in October 1988 we found sections of rocky shore with reasonable densities of most of the rocky-shore littorines. At Pilgramunna, Osprey Bay, and Yardie Creek, respectively, we censused four, five, and five adjacent 1-m-wide transects that included the upper 1.5-m portion of the step in the rock and extended up to 2 m along the horizontal rock at the top of the step.

Species

We studied four species of mangrove littorines that all have northern distributions within Australia and are close to their south-

ern limit of distribution at Ningaloo Marine Park. *Littoraria cingulata* (Philippi, 1846) and *L. sulculosa* (Philippi, 1846) are Western Australian endemic species and are difficult to distinguish as small recruits, although diagnostic features are obvious in larger individuals. *L. filosa* (Sowerby, 1832) and *L. scabra* (Linnaeus, 1758) have widespread Indo-West Pacific distributions. The mangrove snails have relatively thin shells, which are delicate compared with their robust rocky-shore counterparts. *L. filosa* and *L. scabra* are ovoviparous with planktotrophic larvae (Reid 1989), and because *L. cingulata* is in the same subgenus (Littorinopsis), it is assumed to be ovoviparous too (Reid 1986). *L. sulculosa*, in the subgenus Palustorina, is assumed to have oviparous development (Reid 1986). Most individuals of all four species live on the twigs and small branches of the mangrove trees, but *L. filosa* lives also on leaves, and *L. scabra* live also on large branches.

Four species of littorines were common at the rocky-shore sites. *Nodilittorina australis* (Gray, 1826), another western species endemic to Western Australia, were found at Mandu Mandu in two morphological forms previously recognized as two species (Rosewater 1970) but which clearly are a single species with a plastic morphology (Johnson & Black 1999). The three other species, *N. millegrana* (Philippi, 1848), *N. trochoides* (Gray, 1839), and *L. undulata* (Gray, 1839), live in northern Australia and the Indo-West Pacific (Rosewater 1970, Wilson 1993). A fifth rocky-shore species, *N. unifasciata* (Gray, 1826), which has a southern distribution in Australia, appeared sporadically at some of our sites but was too rare to provide consistent information. The four abundant species were easily distinguished by the external characteristics of the shells and by their distribution on the shore. *N. trochoides* appeared on the high part of the shore, and *N. millegrana* appeared on the low part. *N. australis* occupied intermediate levels of the shore, overlapping with the highest *N. millegrana*. *L. undulata* also occurred at intermediate levels of the shore, overlapping with the lowest *N. trochoides* and the highest *N. australis*. *N. millegrana* and *N. trochoides* spawn single ova in pelagic cupola capsules, and their development is planktotrophic; *L. undulata* is identical except for having a biconvex disk capsule (Reid 1989). *N. australis* is thought to be oviparous (Rosewater 1970). Thus, in all eight species studied, recruitment was from planktonic larvae.

Sampling Scheme

Sampling was conducted in two phases. The first phase emphasized the spatial and temporal variability of the recruits. Between October 1988 and June 1991 we made twelve, approximately quarterly, visits to our rocky-shore sites in which we estimated the abundances of recruits by collecting them from the sites. We began collecting the recruits from the mangrove trees in March 1989 so that the number of censuses of the mangrove littorines was only eight. We did not return the recruits from the rocky shores for any of the twelve censuses, but we did return the recruits on the mangroves in the last three censuses. The other phase of our sampling involved censuses of all littorines before (June 1988, rocky shore only) and after (June in 1991, 1993, 1995, and 1999, rocky shore and mangroves) our intensive censuses of recruits. Although we had censuses of total snails for some of the visits when we removed recruits, we excluded those censuses from analyses that quantified the spatial and temporal variability of the total populations. For the total populations, we had five censuses of rocky-shore total populations and eight censuses of mangrove unaffected by our collections.

Our basic sampling unit at the mangrove site was a mangrove tree, but there was further structure to the data because there were four groups of trees. At the northern end of Mangrove Bay, trees A, B, and C formed one group and were 50 m offshore from two pairs of trees (D and E, and F and G). Trees in each of these three groups had branches that overlapped. The fourth pair of trees, H and I, were separated by about 200 m and were at the southern end of the bay about 1.5 km from the other groups. Thus, trees were nested within groups and represent spatial heterogeneity at the smallest spatial scale at which we sampled. Groups of trees represent spatial heterogeneity within Mangrove Bay. When we conducted censuses of recruits or of total snails during low tides, we made careful searches of all the surfaces of all the leaves, twigs, small stems, branches, and main trunks of the trees, using both sight and touch, and collected each individual. We made some preliminary detailed recordings of the microhabitats in which each individual snail was located, which helped us appreciate exactly where snails lived on the mangrove plants. We usually searched the trees twice, looking at the leaves and branches from different directions, but our searches were not 100% efficient, as judged by our discovery of a few missed snails when we returned snails after measuring them. We measured the length of each snail to the nearest 0.1 mm with calipers and returned all the snails, usually on the next day. Wetting the snails made them active, and they readily clung to or stuck with mucus to surfaces on which they were placed.

At the four rocky-shore sites, our basic sampling unit was a 1-m-wide strip of rocky shore that spanned the vertical range of distribution of the littorines. At all the sites the replicate strips were adjacent to each other and were nested within the site; they were therefore comparable to the trees sampled for mangrove littorines. The sites sampled for rocky shore littorines were separated by 5 to 22 km so that the spatial heterogeneity represented by sites was at a much larger scale than for groups of trees at Mangrove Bay (Fig. 1). Our censuses were conducted at low tide, using fine forceps to pick up the tiny recruits and to extract snails from crevices. We searched each area from different angles, attempting to find all the snails. We measured the snails by shaking them through a series of sieves with holes in 0.5-mm increments, from 2.0–10.0 mm, and counting the numbers retained on each sieve. We returned the snails, except for the recruits from sieves 3.5 mm and smaller, in the quarterly samples to the vertical section within each 1-m strip from which they had been collected. We wet the snails to activate them and splashed water on the shore so that the snails could reattach themselves before the next high tide.

Statistical Analysis

Our sampling scheme involved three main factors, representing spatial and temporal variation. The basic experimental unit was a mangrove tree or a 1-m-wide strip of rocky shore. These were nested within group (for the mangrove littorines) or site (for the rocky shore). We repeatedly sampled the same experimental units at different times (quarters for the recruits and years for the total populations), so the model also included a term representing the interaction between the spatial and temporal terms. This was a repeated measures design with between-subjects terms involving the spatial factors and the within-subjects terms involving the temporal factor and the interaction term. In our view, all the factors in this sampling design were random because we were not concerned with the particular sites or specific times of sampling. Table

TABLE 1.

Model for the analysis of variance for the sampling scheme.

Source	G	Degrees of freedom MC	MR	RSC	RSR	Components in the test denominator
Between subjects						
S	a − 1	3	1	3	3	residual + subject (S) + T × S
Subjects (S)	a (r − 1)	5	3	13	13	residual
Within subjects						
T	b − 1	7	7	4	11	residual + T × S
T × S	(a − 1) (b − 1)	21	7	12	33	residual
	residual a(r − 1) (b − 1)	35	21	56	143	

The model involved a levels of the random spatial factor (S) and b levels of the random temporal factor (T), with repeated measures taken on r replicates of the experimental units (subjects) nested within the spatial factor. The degrees of freedom are shown for the mangrove (M) and rocky shore (RS) samples for each of the complete censuses (C) and the counts of recruits (R) as well as the general case (G) for a balanced design. The components of the test denominator are the synthesis produced by the statistical program JMP (SAS Institute, Cary, NC); each analysis will have particular coefficients associated with each term.

1 shows the terms of the model and the components in the denominator of the F test for each term. We used the Fit Model procedure of the JMP Statistical Discovery Software (SAS Institute, Cary, NC) to perform the repeated measures analysis of variance (ANOVA) of the model with all the factors as random and to calculate the variance component for each term in the model. We present these variance components as a percentage of the total variance and show the statistical significance of the terms.

Survivorship and Growth Rates

On eight occasions for the mangrove snails and on five occasions for the rocky shore snails we marked groups of snails with Humbrol Enamel model paint, using different colors as a code for the time of marking. After drying the shell, we painted the margin of the aperture, as illustrated in Behrens Yamada (1989, Fig. 2). For the recaptured snails the position of the paint delineated the margin of the aperture permitted measurement of the size at which the snail had been painted. We used the total length to the nearest 0.1 mm at painting and at recapture and the time interval in years (t) between marking (coded by the color or paint) and recapture to provide estimates of von Bertalanffy growth equations as overall summaries of growth. We used the nonlinear fit procedures of the JMP software to obtain estimates of the von Bertalanffy parameters: $L\infty$ (asymptotic size) and k (growth coefficient) in fitting the function, increment in length = $[L\infty - \text{initial length}][1 - e^{(-kt)}]$.

We used the regression of the natural logarithm of numbers of marked snails recaptured on time in years after initial painting to estimate the instantaneous rate of mortality and converted that to finite annual rate of survival. Similar methods of marking littorines and calculating growth and survival have been used and described in detail by Hughes and Roberts (1981).

RESULTS

Spatial and Temporal Variation of Abundances of Mangrove Littorines

During the eight censuses of the total numbers of littorines on individual trees, each species was absent from each of the trees at least once, except for *L. filosa*, whose lowest abundance was as a single individual on tree H. *L. sulculosa* was completely absent from trees B, E, and F and was the least common of all the species on the other trees, averaging a maximum of only 4.5 on the six occupied trees. In contrast, *L. filosa* was usually the most abundant, with a maximal abundance of 349 on tree H and an overall average maximum abundance on all trees of 130.5. Comparable figures were 165 and 50.2 for *L. scabra* and 73 and 16.7 for *L. cingulata*. Therefore, there was considerable spatial and temporal

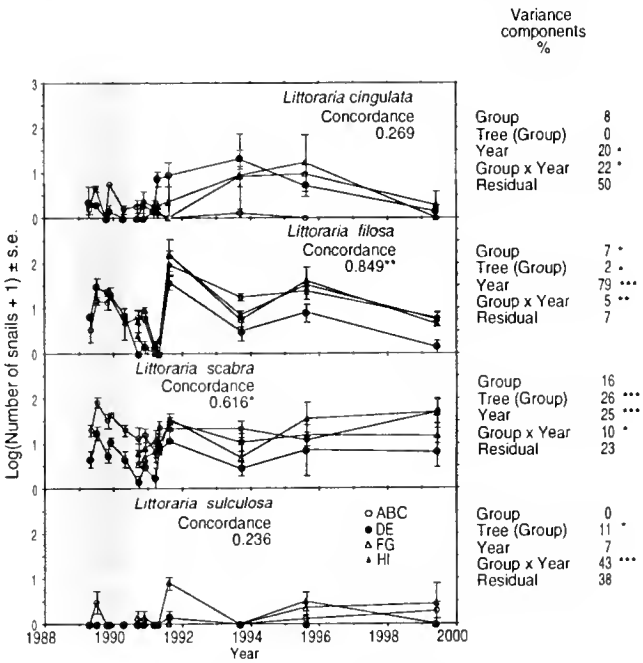

Figure 2. Mean abundance (±SE) of four species of littorines on four groups of mangrove trees at Mangrove Bay, on twelve occasions between April 1989 and June 1999. The shaded area of the plots represents times when our collection of recruits influenced the abundance of the snails; these times are not included in the analyses of concordance or the variance components. Groups ABC and DE were censused at all the times, but censuses began in August 1990 for groups FG and HI. Lines join the points for each of the four groups. The total numbers of snails censused were 308 *Littoraria cingulata*, 1,963 *L. filosa*, 1,869 *L. scabra*, and 47 *L. sulculosa*. The coefficients of concordance among species within a group of trees were ABC 0.069; DE 0.496; FG 0.449; HI 0.611. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$ for coefficients of concordance and F tests associated with the terms in the analysis of variance.

variability in the abundances of these snails in our sample of mangroves.

Figure 2 shows the average of the logarithms of the counts of snails of each species for the four groups of trees and provides two quantitative summaries of the spatial and temporal variability for the eight censuses when we were not collecting and removing the recruits (unshaded portion of the figure). The first summary is the value for the Kendall coefficient of concordance (Siegel 1960). For the values listed for each species, this represents the correlation between the averages for each group of trees across the eight censuses and therefore indicates whether the abundances on the four groups of trees changed in concert. The abundances of *L. filosa* and *L. scabra*, the two most abundant species, were significantly concordant judged by this statistic and as indicated by the few crisscrosses of lines joining the census values for each group of trees. For the values for concordance listed in the caption of Figure 2 for the four groups of trees, which tests whether the four species fluctuated in concert over time in each group, only trees H and I showed significant concordance. Thus, these analyses indicated that *L. filosa* and *L. scabra* had synchronized temporal fluctuations across the 1.5 km spanned by the four groups of trees, but because the species were not concordant in three of the four groups, the temporal patterns were not consistent among species.

The second summary of temporal and spatial variability in Figure 2 is the variance components. The percentage values shown for each term in the model of our sampling scheme sum to 100% for each of the species. The clearest result was for *L. filosa*, which extends the result from the analysis of concordance. The term for year had the largest variance component at 79%, indicating that temporal variation contributed most of the variability, and the term for group × year was low, indicating that the four groups varied in concert over time. The variance components for *L. scabra* also had a relatively high value for year and a relatively low value for group × year, as expected from the significant index of concordance, but the three other terms in the model contributed substantially to the total variance as well, represented in Figure 2 by the separation of the group averages at each time, the crisscrossing of the lines, and the large standard errors. The variance components for *L. sulculosa*, the least abundant species, had the most variability explained by the group × year term and the residual term. *L. cingulata* differed from the other species in having the residual term contribute most of the variation (represented on the figure by the standard errors associated with each point, which indicates the tree × group [tree] × year interaction). The final important point about the variance components is that the distribution of variance among the terms of the model was different for each species, indicating that, at the scales represented by the spatial distribution of the groups of trees and by the trees within groups, the abundances of four species varied independently.

Figure 2 shows information for five censuses not included in the previous analyses because we collected and retained the recruits (grayed area of figure). One striking feature of these data for the two most abundant species, *L. filosa* and *L. scabra*, was the general decline in the average total abundance in the groups of trees ABC and DE. This suggests that the supply of new individuals may determine abundance in these populations and focuses attention on the dynamics of recruitment.

The detailed information on the abundance of recruits came from eight censuses of recruits on five trees. Because the recruits of *L. cingulata* and *L. sulculosa* are indistinguishable, they are pooled in this set of data, which therefore consists of 120 observations. Recruits were rare, because 105 of these counts were zero. Recruits of *L. scabra* had the highest average of 6.8 per tree, followed by *L. cingulata/sulculosa* with 2.0 and *L. filosa* with 0.4, but as Figure 3 shows, there was considerable spatial and temporal variability.

Even though the coefficients of concordance were large, the power of this nonparametric test was low, so none of the coefficients were statistically significant (Fig. 3). However, the patterns suggested by the coefficients of concordance were revealed clearly by the variance components. The temporal coherence suggested for the species, except for *L. filosa*, were indicated by high contributions to variance by the quarter term in the analysis of variance and by the group × quarter term for the pooled species. The two groups of trees contributed substantial variance too, with the offshore group ABC having more *L. scabra* and fewer *L. cingulata/sulculosa* than the inshore group DE. The variance components for *L. filosa* were dominated by the contribution of the residual term, indicating the difficulty of detecting spatial or temporal pattern for the few recruits. In short, although the spatial and temporal scales differed between the data for recruits and total snails, the nature of the spatial and temporal variability was similar: the recruits of the species varied independently of each other over space and with time, and low numbers of recruits appeared sporadically.

Spatial and Temporal Variation of Abundances of Rocky-Shore Littorines

L. undulata was the least abundant of the four species on the rocky shores, averaging about six individuals per 1-m strip across the four sites and five censuses not influenced by our collection of

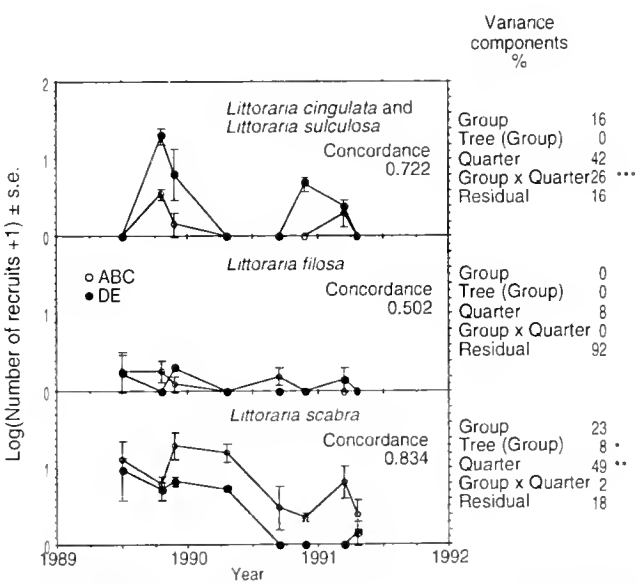

Figure 3. Mean abundance (±SE) of recruits of littorines on two groups of mangrove trees at Mangrove Bay on eight occasions between June 1989 and March 1991. Lines join the points for each group. The total numbers of snails censused were 79 *Littoraria cingulata* and *L. sulculosa*, 16 *L. filosa*, and 273 *L. scabra*. The analyses of concordance and variance components use all the data shown in this figure. The coefficients of concordance among species within a group of trees were: ABC 0.429 and DE 0.410. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$ for coefficients of concordance and F tests associated with the terms in the analysis of variance.

recruits. It was absent from Pilgramunna on all five censuses and from Osprey Bay on one census. *N. trochoides* and *N. australis* were the most abundant, averaging 66 and 55, respectively, and *N. millegrana* was intermediate at 16. Mandu Mandu had the highest average abundance per 1-m strip of each species and the highest abundance of snails summed over all species on each date.

As judged by the coefficients of concordance in Figure 4, of the four species only *N. trochoides* had significant temporal patterning among sites, and only Mandu Mandu had significant temporal patterning among species. However, the variance components from the ANOVA revealed one consistent pattern among the species: the site term formed the largest percentage for all four species, and more than half for *N. trochoides* and *L. undulata*, reflecting the consistently greater abundance at Mandu Mandu. Furthermore, for these two species, the year and year × site components together were only 18%, indicating considerable spatial and temporal consistency despite statistically significant interaction terms, as seen by the different average abundances at the sites and lack of crisscrossing of the lines joining the data points. A second consistent feature of the variance components was the low percentage associated with the strip within site term, that is, between the adjacent 1-m-wide strips of the shore. *N. australis* had the largest value at 14%, and *L. undulata* the lowest at 0%. In brief, for the rocky shore littorines, large-scale (between-sites) spatial variability dominated and, except for *N. australis*, exceeded temporal variability.

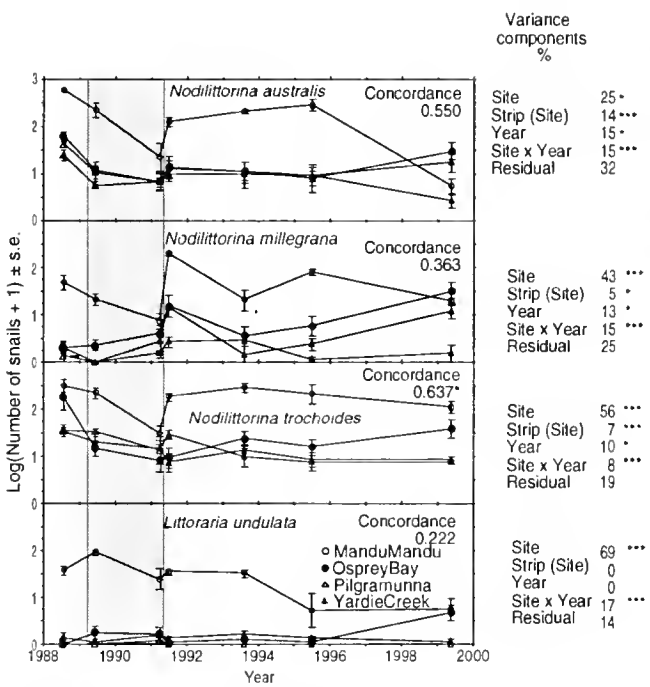

Figure 4. Mean abundance (±SE) of four species of littorines at four rocky shores in Ningaloo Marine Park on seven occasions between June 1988 and June 1999. The shaded area of the plots represents times when our collection of recruits influenced the abundance of the snails; these times are not included in the analyses of concordance or the variance components. Lines join the points for each of the four sites. The total numbers of snails counted in these censuses were 6,506 *Nodilittorina australis*, 1,856 *N. millegrana*, 7,905 *N. trochoides*, and 795 *Littoraria undulata*. The coefficients of concordance among species within sites and the number of strips (in parentheses) were Mandu Mandu 0.536 ($P < 0.05$) (3); Osprey Bay 0.312 (5); Pilgramunna 0.378 (4); and Yardie Creek 0.437 (5). *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$ for coefficients of concordance and F tests associated with the terms in the analysis of variance.

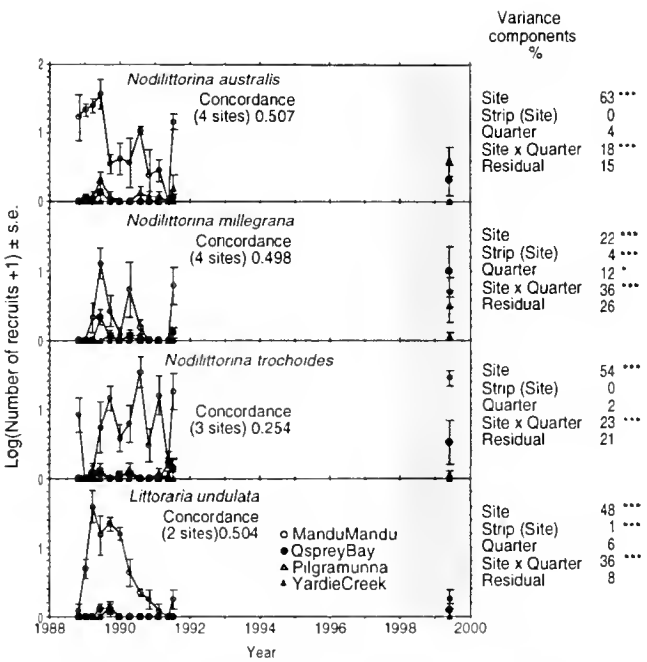

Figure 5. Mean abundance (±SE) of recruits of four species of littorines at four rocky shores in Ningaloo Marine Park on twelve occasions between October 1988 and July 1991. The shaded area of the plots, a census in 1999, was not included in analyses of concordance or variance components. Lines join the points for each of the four sites. The total numbers of snails counted in censuses were 514 *Nodilittorina australis*, 126 *N. millegrana*, 440 *N. trochoides*, and 389 *Littoraria undulata*. The analyses of concordance and variance components use all the data shown in this figure. The coefficients of concordance among species within a site and the number of strips (in parentheses) were Mandu Mandu 0.415 (3), Osprey Bay 0.344 (5), Pilgramunna 0.191 (4), and Yardie Creek 0.143 (5). *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$ for coefficients of concordance and F tests associated with the terms in the analysis of variance.

The gray area of Figure 4 indicates the censuses influenced by our collection of recruits, and as for the mangrove littorines, the downward sloping lines for the total abundances suggested that the removal of recruits may have led to decreases in populations for some of the species at some of the sites. The abundances of these recruits are shown in Figure 5 for censuses during late 1988 to mid 1991.

In the twelve censuses of the four species at the four sites, all species had no recruits on at least one of the quarterly sampling times, and for *N. australis* and *N. millegrana* recruits were never recorded at Yardie Creek; in all, 120 of the 192 observations were zero. Averaged over all sites and quarters, the average abundance of recruits ranged from 2.5 for *N. australis* to 0.6 for *N. millegrana*, with *N. trochoides* and *L. undulata* intermediate at 2.15 and 1.90 per 1-m-wide strip, but these values are largely influenced by the high recruitment at Mandu Mandu (Fig. 5).

None of the coefficients of concordance was statistically significant, and all were less than 0.55, so there were no strong temporal patterns of abundance. As for the analysis of the variance components of the total abundances, the percentage for the site term was about 50% for all species except *N. millegrana*, so spatial variability, at the scale of tens of kilometers, dominated the abundance of recruits (Fig. 5). The abundance of recruits was greatest

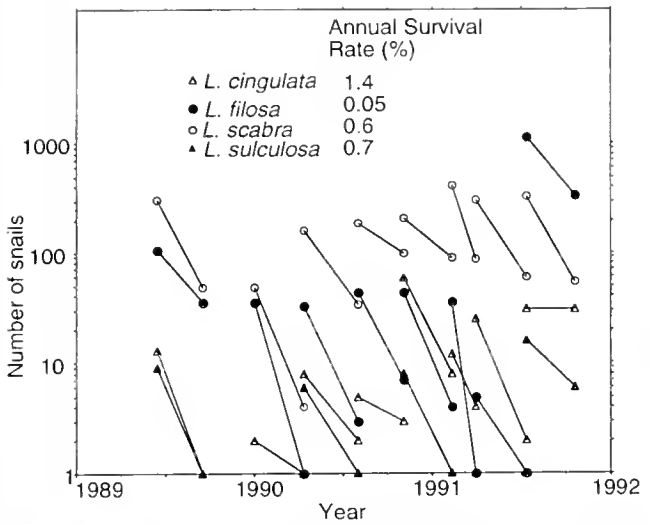

Figure 6. Numbers of snails painted at the beginning of an interval joined by straight lines to the numbers recaptured after a short interval, shown separately for the four species of mangrove snails, summed over all trees. The total numbers of individuals initially painted and the numbers of intervals providing data were 1,265 *Littoraria cingulata* (seven intervals), 2,488 *L. filosa* (eight intervals), 2,827 *L. scabra* (eight intervals), and 1,155 *L. sulculosa* (five intervals). The means and standard errors of [ln(painted) – ln(recaptured)]/[interval in years], which convert to annual survival rate as a proportion of one for the species in the same order, were −4.25 ± 1.12, −7.62 ± 1.29, −5.07 ± 0.75, and −5.02 ± 1.00.

by far at Mandu Mandu, in line with that site having the highest total abundance of snails during our entire study (Fig. 4). Furthermore, as for the total snails, the term for strip within site was the smallest, never more than 4%, emphasizing spatial consistency of adjacent strips within each site. For all species of recruits, the variance component for site × quarter term ranked second largest, ranging from 18 to 36%, indicating that the temporal pattern of recruitment differed among sites. As can be seen in Figure 5, the abundance of recruits of all species varied considerably and with different temporal patterns at the Mandu Mandu site. In summary, gains at an instant in time to the populations of rocky-shore snails were small in comparison with the total numbers, spatial variability was great, and temporal variability differed among species.

Survival and Growth of Littorines at Ningaloo Marine Park

The mark and recapture of painted snails provided quantitative estimates of rates of loss from the populations. We discovered that painted snails disappeared quickly from the mangrove trees, and our data consist of eight sets of painted snails observed between adjacent samplings (Fig. 6). Because the values are plotted on a logarithmic scale, the slopes of the lines connecting the numbers marked and recaptured for the groups of snails reflect the instantaneous rates of mortality, which we calculated using natural logarithms and then converted to estimates of annual rate of survival, whose averages are indicated in Figure 6. Clearly, the painted snails, which were mostly large snails rather than small recruits, disappeared at a very high rates so that the annual survival rates were low, with *L. cingulata* having the greatest survival rate at 1.4% and *L. filosa* the least at 0.05% per year. Besides being low, these estimates are also variable, with the instantaneous rates + 1 SE converting to 4.4, 0.2, 1.3, and 1.8% for *L. cingulata*, *L. filosa*, *L. scabra*, and *L. sulculosa*, respectively.

Figure 7 shows the survival of painted snails on the rocky shores. Like its congeners on mangroves, *L. undulata* had a low annual rate of survival of 3%. Similarly, our estimate of survival for the species lowest on the shore, *N. millegrana*, was low at 1%. We rarely recaptured any of these snails, except for those that we had painted as small recruits. On the other hand, *N. trochoides*, from high on the shore, was a survivor; its annual rate of survival was estimated as 24%, and we kept track of some individuals for 4 y before they disappeared. *N. australis* had an intermediate rate at 14%.

All our estimates of survival probably underestimate the true survival because the snails could lose their paint mark. Our census data during the period when we collected recruits provided a second estimate of survivorship, under the assumption that our collections stopped all input to the populations during that time, so that the populations lost snails only due to mortality. The analyses of the regression of ln(abundance) on date in years and the conversions to annual rate of survival in Table 2 show that the estimates were substantially higher than those calculated from the mark and recapture data for both the mangrove and rocky-shore snails but suffered from imprecision in the estimate of the slopes due to the small number of censuses conducted (all the rocky-shore species) and a lack of a distinctly linear decline (*L. cingulata*, *L. filosa*, and *L. undulata*). The estimate for *N. millegrana* is probably inappropriate for the reason stated earlier: This species never had any marked animals survive for more than a year. However, within habitats these estimates probably provide an indication of relative rates. Low survival rates have implications for other features of the life history of these snails, and the recaptured snails provided estimates of rate of growth because we could measure the size at which they were painted as well as size at recapture.

Table 3 shows a summary of the information on growth rates in terms of the fitted parameters for the von Bertalanffy growth equation. The thin-shelled mangrove littorines all had asymptotic sizes ($L\infty$) between 23 and 28 mm, considerably larger than the thicker-

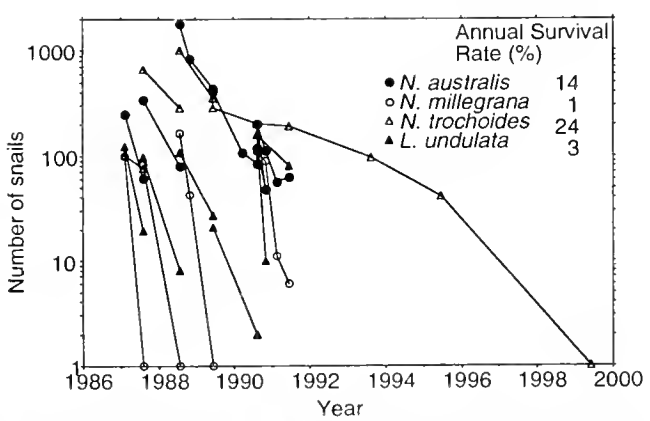

Figure 7. Numbers in groups of snails of the four species of rocky-shore littorines known to be painted at seven times and followed over at least one time interval at Mandu Mandu. Lines join the data for each group of snails. The total numbers of individuals initially painted and the numbers of intervals providing data were 3,174 *Nodilittorina australis* (seven intervals), 570 *N. millegrana* (six intervals), 2,452 *N. trochoides* (10 intervals), and 743 *Littoraria undulata* (seven intervals). The means and standard errors of [ln(painted) – ln(recaptured)]/[interval in years], which convert to annual survival rate as a proportion of one for the species in the same order, were −1.98 ± 0.26, −4.83 ± 1.15, −1.44 ± 0.80, and −3.36 ± 0.96.

TABLE 2.

Estimates of annual rate of survival derived from the slope of regression of natural logarithm of abundance of littorines (Y) on year (X) during the interval when recruits were removed regularly from the populations on trees A to E in Mangrove Bay and from the populations at Mandu Mandu.

Species	Number of censuses	Slope ± Standard Error (Probability, b = 0.0)	Coefficient of determination	Annual rate of survival (%)
Littoraria cingulata	4	−1.920 ± 1.773 (0.47)	0.75	14.7
Littoraria filosa	4	−1.561 ± 0.985 (0.25)	0.75	21.0
Littoraria scabra	4	−1.428 ± 0.578 (0.13)	0.73	24.0
Nodilittorina australis	3	−1.063 ± 0.030 (0.02)	0.99	20.1
Nodilittorina millegrana	3	−0.711 ± 0.109 (0.10)	0.98	49.1
Nodilittorina trochoides	3	−0.900 ± 0.160 (0.11)	0.97	40.7
Littoraria undulata	3	−0.173 ± 0.389 (0.73)	0.17	84.1

shelled rocky-shore snails with values between 10 and 18 mm. With the exception of *N. millegrana*, the rocky shore snails had lower growth rate coefficients (k) than the mangrove species. *L. filosa* and *N. millegrana* stood out as having substantially greater rates of growth than any of the other species.

Figure 8 provides a visual summary of the information about growth with plots of the average size at age predicted by the von Bertalanffy growth equations. The steepness of the rise of the curves reflects the magnitude of the growth coefficient (k), the elevation of the curves represents the asymptotic size ($L\infty$), and the curves for *L. filosa* and *N. millegrana* terminate at the time at which the predicted asymptotic length is reached (greater than 10 y for all the other species). There are three groups of growth curves in Figure 8: The three mangrove species grew fastest and reached the largest sizes, the high-shore *N. trochoides* grew at the slowest rate and reached only moderate size, and the other three rocky shore littorines grew at about the same intermediate rate but reached different asymptotic sizes. As an additional summary of the information on growth, Figure 8 shows the number of years at which the growth equation predicted that each species would reach half the asymptotic size, which is probably a biologically relevant indication of the demographic processes in these populations. By this measure, *L. filosa* and *N. millegrana* were the most ephemeral species because they were predicted to reach half size in less than 0.5 y. The other two mangrove species, *L. cingulata* and *L. scabra*, would reach that size in less than 1 y, and *L. undulata* and *N. australis* would take about 1.25 y. *N. trochoides* was predicted to take nearly 3 y to reach half size.

TABLE 3.

Estimates of the parameters of the von Bertalanffy growth equation* for the eight species of littorinid snails studied at Ningaloo Marine Park.

Habitat and species	Sample size	$L\infty \pm SE$	k ± SE
Mangroves (all trees)			
Littoraria cingulata	41	24.1 ± 3.85	0.72 ± 0.227
Littoraria filosa	340	23.9 ± 0.67	1.96 ± 0.183
Littoraria scabra	314	28.0 ± 0.91	0.89 ± 0.098
Rocky shore (Mandu Mandu)			
Nodilittorina australis	465	14.6 ± 0.11	0.65 ± 0.030
Nodilittorina millegrana	88	10.1 ± 0.61	1.30 ± 0.238
Nodilittorina trochoides	632	13.1 ± 0.27	0.23 ± 0.015
Littoraria undulata	36	18.4 ± 0.91	0.51 ± 0.096

* $L_t = L\infty (1 - e^{(-kt)})$, where $L\infty$ = asymptotic size, k = growth coefficient, and t = age in years.

Most of the records of growth for the mangrove littorines came from the groups marked in October 1991, but data from all intervals were included. The records for the rocky shore came from groups of snails initially marked in the winters of 1988 and 1989 and recaptured after one year, except or *Nodilittorina millegrana* for which we had recaptures in August after marking a cohort in November the previous year. Snails that survived for two or more intervals contributed growth information for each interval.

Covariation of Population and Life History Characteristics

The simultaneous investigations of eight species of littorines allow tests for strong patterns of covariation in the estimated demographic and life history parameters. The population characteristics available for seven of the species were the coefficients of variation for the abundances (measured on a logarithmic scale) of total snails and of recruits, and the sum of the variance components

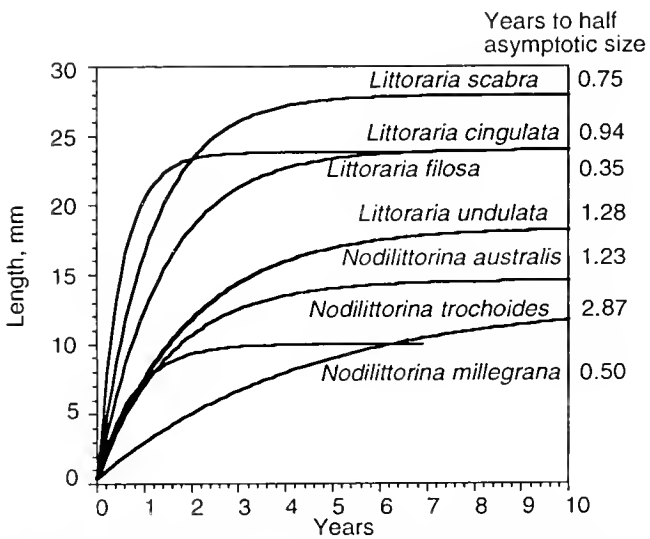

Figure 8. Predicted size-at-age from von Bertalanffy growth equations for three species of littorines from Mangrove Bay and four species from Mandu Mandu along with the age at which the snails are predicted to reach half their asymptotic size. The plots for *Littoraria filosa* and *Nodilittorina millegrana* end at the time at which the equation predicts that they would reach their asymptotic size; the others take longer than 10 y. The parameters of the von Bertalanffy equation, asymptotic size, $L\infty$, and growth coefficient (k) are shown in Table 3.

for temporal and temporal × site terms. The life history characteristics were the estimates of the growth rate constant and the time to half asymptotic size from fits to the von Bertalanffy growth equation and the two sets of estimated annual survival rate. Two patterns stood out, as shown in Figure 9.

The first pattern was a negative relationship between the coefficient of variation of the abundance of total snails and the mean of the abundance of recruits, with the position of the points for the rocky-shore species above and to the right of the mangrove species (Fig. 9a). On the rocky shore, *N. trochoides* and *N. australis* had higher recruitment and the least variable population of snails, *L. undulata* had the fewest recruits and the most variable population, and *N. millegrana* was intermediate. The pattern for the mangrove snails was less obvious, with *L. scabra* having more recruits and less variable populations than *L. filosa* and *L. cingulata*. These data address the issue of supply-side ecology by pointing to the contribution of abundant recruitment in stabilizing the populations.

The second pattern was a positive association between annual survival rate calculated from the recapture of painted snails and the predictions of years to half asymptotic size (Fig. 9b). There was a significant positive correlation involving all seven species, but it was unclear whether the important grouping of the points was associated with the genus of the snails or with the habitat in which they lived because *L. undulata* from the rocky shore was directly in line with its congeners from the mangroves.

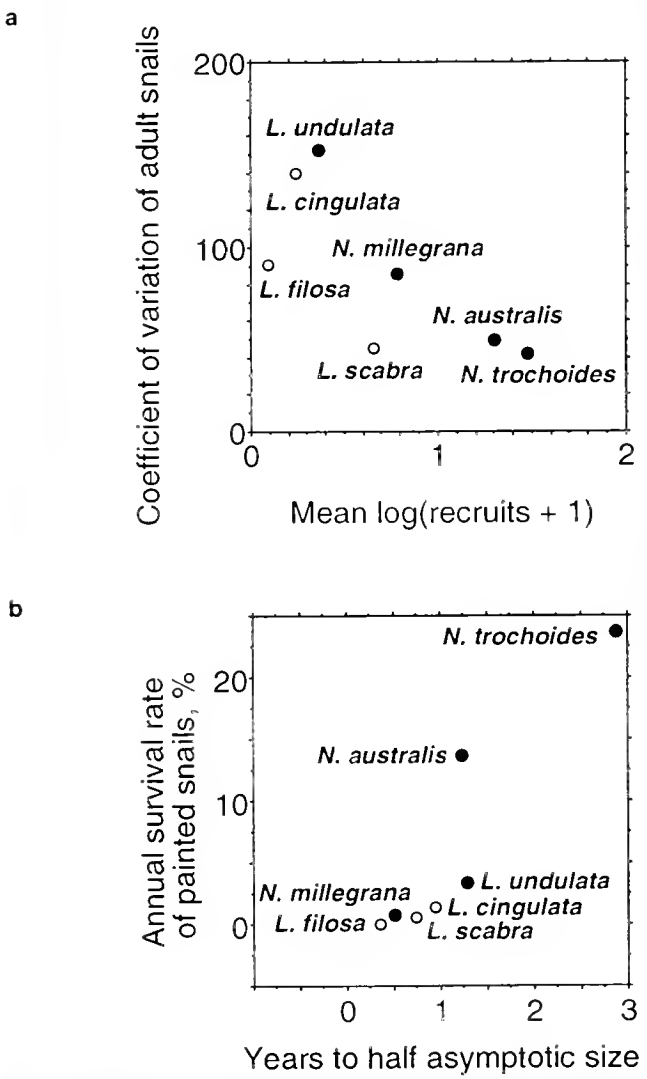

Figure 9. Covariation of demographic and life history characteristics of three species of mangrove littorines and four species of rocky shore littorines at Ningaloo Marine Park. (a) Overall, the coefficient of variation of adult snails and mean log (recruits + 1) had a significant negative correlation ($r = -0.73$), with the correlation coefficient for rocky-shore species higher ($r = -0.97$) and positioned farther to the right than for the mangrove species ($r = -0.70$). (b) Estimates of annual rate of survival of painted snails overall had a significant positive correlation ($r = 0.92$) with the years to half asymptotic size. The correlation coefficients for the three species of *Nodilittorina* and the four species of *Littoraria* were similar ($r = 0.95$ and $r = 0.94$, respectively) as were the correlation coefficients for the three mangrove species and the four rocky-shore species ($r = 0.95$ and $r = 0.91$, respectively).

DISCUSSION

This decade-long study of eight species of littorines at Ningaloo Reef revealed three important features of the population ecology of these species. The most conspicuous feature revealed was that, at the spatial and temporal scales used in this investigation and for the sets of species in the two habitats, the species' abundances varied largely independently, as indicated by the different patterns in the variance components (Fig. 2–5). Thus, even though all the species have planktonic larvae and our sites were spread over only 40 km of coastline, patterns of abundance of all snails and of recruits differed over space and time. There were, however, some conspicuous exceptions to this general feature in some components of the patterns. The abundances of *L. filosa* and *L. scabra* on the four groups of trees did vary in concert over time, but the patterns differed between these species. The differences in abundance of the rocky-shore littorines were dominated by differences among sites because of the consistently high numbers of all snails and of recruits at Mandu Mandu, where the four species also varied in concert. Finally, for total abundances, the variance component at the spatial scale of adjacent sampling units, represented by tree (group) and strip (site), was consistently the lowest for all species except for *L. scabra*, where it was the greatest. However, even these patterns are inconsistent across species, so, overall, patterns of spatial and temporal variability are unique for each species.

The second important feature involving abundances of these littorines was the relation between supply of recruits and the variability of the total abundances, especially for the rocky-shore species (Fig. 9a): The greater the number of recruits, the less variable was the number of snails. There is another aspect about the importance of the supply of recruits related to the source of the recruits. As can be seen in Figures 2 and 4, all the species except *L. scabra*, *N. australis*, and *N. trochoides* were absent from a group of trees or a site on at least one occasion. Because all the species recovered from being absent at a group or site, recruits must come from elsewhere, emphasizing the role of the planktonic larvae and the open nature of the populations. Furthermore, these absences of species from some sites at some times point out how misleading surveys at single sites and times could be about the distribution and abundance of these species. As a case in point, Reid (1986) specifically indicated that *Littoraria* spp. were not found at Mangrove Bay, presumably on the basis of surveys when the snails were scarce.

The third aspect about population ecology concerns the diversity of turnover rates exhibited by the eight species of littorines studied at Ningaloo Reef (Fig. 9b). The dynamics of populations of

L. filosa and *N. millegrana* operated much faster than other species, with fast rates of growth combined with low rates of survival suggesting an annual life cycle, as has been interpreted for other littorines (e.g., Underwood & McFadyen 1983 for *L. acutispira* and Williams 1992 for *L. mariae*). *N. trochoides* was at the other extreme, with high survival and a slow rate of growth and probably being long-lived like *L. neritoides* (Hughes & Roberts 1981). On average, the mangrove species were more ephemeral than the rocky-shore species, but both groups vary considerably, and it is impossible to determine from our small sample and the ambiguous position of *L. undulata* whether the patterns in Figure 9b are driven by habitat or generic relationship. To our knowledge, this is the first comparative study of demography in species of *Littoraria* and *Nodilittorina*. The variability and the apparent patterns highlight the potential of these snails for testing general ideas about life-history evolution.

ACKNOWLEDGMENTS

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PREDATION BY THE PILE PERCH, *RHACOCHILUS VACCA*, ON AGGREGATIONS OF THE GASTROPOD *LITTORINA SITKANA*

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ABSTRACT The pile perch, *Rhacochilus vacca* (Embiotocidae), is abundant on the Pacific Coast of North America and is known to crush hard-shelled prey with its heavy pharyngeal teeth. We investigated whether pile perch predation has the potential to limit or regulate populations of *Littorina sitkana*, a direct-developing gastropod found on wave-sheltered shores near Bamfield, British Columbia, Canada. Laboratory experiments showed that pile perch required an average of only 19.2 sec (SE = 5.61; $n = 20$) to crush and swallow a large *L. sitkana*, which resulted in consumption rates averaging 33.3 (SE = 6.27; $n = 4$) large snails per day. The snails had no size refuge from predation by adult fish because even small fish (fork length 21 cm) could crush the largest *L. sitkana* present on the shore (>11 mm shell width). Indeed, some fish showed a significant preference for large snails (shell width >6.3 mm) over small snails (3.35 mm < shell width < 4.0 mm). Our 1998 observations with SCUBA during daytime high tides showed that the density of pile perch foraging in the intertidal averaged 0.119 (SE = 0.0205; $n = 20$) individuals per square meter (estimated fork lengths 5–40 cm). However, the intertidal distribution of pile perch that were actually consuming prey was highly aggregated. In the field, we investigated whether predation by this fish on snails deployed onto boulders was density dependent. The fish swam parallel to the shore and located and consumed 40% of the patches of *L. sitkana* that we deployed ($n = 70$) within 50 min. This foraging behavior resulted in density-dependent predation on the deployed snails in only one of the three tidal cycles (or 2 of the 12 days) of our experiment. We offer several proximate reasons for the low frequency of density-dependent predation found in this study and conclude that the pile perch may not be an important regulating factor for *L. sitkana* populations at this site at the present time. However, the very high predation rates we observed suggest that this predatory fish is an important limiting factor at this site.

KEY WORDS: aggregative response, limiting factor, population regulation

INTRODUCTION

The factors that determine the distribution and abundance of marine invertebrates are still not well understood. Predation is known to be important in limiting the abundance of littorinid gastropods on rocky shores. Paine (1994) reviewed examples where predators had been excluded from rocky shores and concluded that such exclusions usually resulted in dramatic increases in the abundance of the competitive dominant. Many of the classic exclusion experiments he reviewed have involved slow-moving predators, such as gastropods or starfish. However, slow-moving predators are unable to move rapidly to high-density aggregations of their prey and are generally less tolerant to emersion than their prey (Newell 1970). As a result, they usually have a very local effect at the lower end of the intertidal distribution of their prey. In contrast, highly mobile predators, such as crabs and fish, can forage throughout the intertidal during a single high tide period and have been observed to feed more heavily on high-density aggregations of their prey (Boulding & Hay 1984, Behrens Yamada & Boulding 1996). Sinclair (1989) argues that only density-dependent factors can provide the negative feedback that can regulate a population at equilibrium. Consequently, highly mobile predators may be disproportionately important as regulating factors on rocky intertidal shores.

Two highly mobile predators, crabs and fish, can prey very heavily on littorinid gastropods on wave-sheltered shores of the northeastern Pacific, and it is not clear which predator type is more important. McCormack (1982) deployed *Littorina sitkana* into fenced areas of the intertidal and observed predation rates of up to 54% per daytime high tide period, which she attributed entirely to predation by pile perch (*Rhacochilus vacca*). Behrens Yamada and Boulding (1996) tethered *L. sitkana* in the intertidal zone and found predation rates of up to 77% per high tide period. They recovered diagnostic shell fragments that had been peeled by crabs from 40% of the dead snails of shell length 15–17.5 mm. Boulding et al. (1999) found predation rates on tethered *L. sitkana* to be up to 25% per high tide period and used shell fragments to attribute at least 52% of the predation of their largest size class to predation by crabs. The daily rates of predation reported above are too high for any *L. sitkana* population to sustain given their known reproductive rates (Behrens Yamada 1989). We hypothesize that these high predation rates represent a strong density-dependent response to local high-density patches of prey and present experiments here to test our hypothesis.

Foraging excursions into the intertidal during high tide have been well documented for large subtidal crabs such as *Cancer productus* (Boulding & Hay 1984, Robles et al. 1989, Behrens Yamada & Boulding 1996, Boulding et al. 1999). There is experimental evidence that predation by this crab species can be density dependent (Boulding & Hay 1984).

However, much less is known about the foraging behavior of the pile perch other than its extreme morphological specializations for crushing hard-shelled prey (Brett 1979). One of the major components consistently found in stomach analyses of pile perch are gastropod molluscs (Ellison et al. 1979, Haldorson & Moser 1979, Hueckel & Stayton 1982, Laur & Ebeling 1983, Stouder 1987). The only other surf perch that is common in the intertidal zone on the west coast of Vancouver Island and is known to eat gastropods is the striped perch (*Embiotoca lateralis*) (Lamb & Edgell 1986, E.G. Boulding Pers. Comm.). Striped perch stomachs sometimes contain gastropod mollusks, but soft bodied invertebrates such as amphipods, bryozoans, and isopods are much more prevalent (Haldorson & Moser 1979). The different feeding preferences of the two species are correlated with their mouth size and structure. The pile perch has extremely large pharyngeal plates, heavy blunt (pavement-like) pharyngeal teeth, and well-developed associated musculature for specialization in crushing and grinding (DeMartini 1969). The striped perch, however, has only moder-

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ately sized pharyngeal plates; smaller, more pointed pharyngeal teeth; and moderately developed musculature and is specialized for feeding on large whole prey (DeMartini 1969). For these reasons, we decided to focus on determining whether pile perch predation on patches of *L. sitkana* deployed onto a rocky intertidal shore was density dependent.

MATERIALS AND METHODS

Study Area

Our experimental site was located on a wave-sheltered boulder shore in front of Bamfield Marine Station (48°50′, 125°08′) in Barkley Sound, Vancouver Island, British Columbia, Canada. We chose the site because it was one of the sites ("Pebble Beach A," hereafter Pebble Beach) used by McCormack (1982) in her study of the role of pile perch in maintaining shore-level size gradients in *L. sitkana* populations.

Laboratory Feeding Experiments

Our laboratory feeding experiments were conducted in round fiberglass tanks at Bamfield Marine Station. The experimental tanks were either 1.22 or 1.83 m in diameter and were filled with free-flowing sea water to a depth of 0.914 m. The tanks were covered with plywood but were left open along a 20-mm crack to allow for low levels of light. The flow rate of seawater into the tanks was at least 1 l/s, and the water had a temperature of 10 ± 1°C and a salinity of 31‰.

The pile perch were collected by fishing with a hook baited with a whole mussel (*Mytilus trossulus*). Upon landing, the hook was carefully removed with pliers, and the fish was placed in a bucket of sea water and rapidly transported to 2.44-m diameter round holding tanks. The fish were then held for at least a week and maintained on a diet of *M. trossulus*. The total length of the fish collected ranged from 275–330 mm.

L. sitkana were collected from Seppings Island near Bamfield Marine Station. The large size-class of snails was also collected from nearby Second Beach. They were held in perforated containers in free-flowing sea water until used in the experiments. The size of the minimum diameter (shell width) of the snails was determined by passing them through a series of brass soil test sieves stacked so that the mesh size decreased from the top sieve to the bottom sieve.

Three different feeding experiments were conducted using one fish in each experimental tank. In the first experiment (Consumption Amount) we offered each of four pile perch 100 extra-large (>6.3 mm shell width) *L. sitkana* placed at the bottom of the tank. After 24 h we recorded the number of live snails above, at, and below the water line and then removed the live snails and the shell fragments from each tank. This experiment was continued for nine replicate days. We chose not to use repeated measures analysis of variance (ANOVA) because our primary objective was to compare the consumption rates of the different fish rather than trends over time. Instead, we used a one-way ANOVA with FISH as the factor and then used Bonferroni pairwise comparisons to determine whether some individual fish ate more than others. We used version 5 of the statistical package SYSTAT for this and all other statistical analyses (SYSTAT 1992).

In the second experiment (Size-Selection) we offered each of four pile perch 100 extra-large and 100 small (3.35 mm < shell width < 4.0 mm) *L. sitkana*. After 24 h we recorded the number of live snails of each size-class above, at, and below the water line. We then removed the live snails and the shell fragments from each tank. This experiment was conducted for 6 days. To analyze the data we used three-way ANOVA with DAY, FISH, and SIZE as the factors and included only the interaction we thought most important, FISH × SIZE, in our model. One fish did not eat and was excluded from further analysis.

In the third experiment (Consumption Rate) we opened the crack in the plywood cover to 10 cm and offered each of four pile perch 20 large *L. sitkana* (4.75 mm > shell width < 6.3 mm). We then used a stopwatch to record the time it took the fish to crush each snail. We noted that only certain fish would feed while being observed.

Field Transects

In 1998 we deployed two 50-m transect lines at Pebble Beach (below the *Fucus* zone) parallel to the depth contours at 0.8 m and 1.1 m above 0.0 datum (Canadian Hydrographic Services). The transects were surveyed for pile perch and striped perch densities. Counts were done using SCUBA during high tides of 3.0 m or greater such that the upper transect (1.1 m) was in at least 1.9 m of water. Two observers swam along the transect line, one on each side, for about 15 min recording fish densities and size classes within 2 m of their side of the line. Fish size-classes were divided into 5-cm intervals from a minimum length of 5 cm to a maximum length of 40 cm. A total of five surveys were done in July 1998. We did four similar transect surveys between June 28 and July 1, 1999, except that these transect lines were at 0.0 m and 1.3 m above 0.0 datum (Canadian Hydrographic Services). In 1999, we tested how good the divers were at estimating the total length of the fish by placing models of different sizes of fish underwater at distances from 2–6 m from each diver and asking her to estimate its total length.

We estimated the abundance and mean size of mollusc prey at Pebble Beach by counting and measuring the molluscs in five 10-cm × 10-cm quadrats sampled at random along a tape measure placed on the beach, parallel to the water's edge, at low tide. The sampling was repeated at 1.5 m, 2.2 m, 2.8 m, and 3.2 m above 0.0 m datum. Because only one *L. sitkana* was found in the quadrats, an additional search was done to find *L. sitkana* by walking along the entire beach at low tide and turning over rocks for a total of 2 h. The abundances were then compared with estimates of *L. sitkana* density done by McCormack at the same site in 1981.

Field Experiments

In 1999 we used SCUBA to deploy three high-density and three low-density replicate patches of snails each day at high tide for a total of 12 days. A container with either a high-density patch (n = 50 snails) or a low-density patch of *L. sitkana* (n = 5 snails) was chosen at random from the mesh bag carried by the divers. The divers opened the container, and the snails were placed on boulders along the 1.3-m depth transect line. We chose boulders that were flat on top, about 0.3–0.6 m in diameter and 0.3–0.5 m high, and cleared them of macroalgae. This clearing was done to reduce variance among replicates caused by the snails hiding more effec-

tively on some rocks than on others. After 40–50 min the divers returned to the boulder, collected all the live snails and shell fragments from the boulder and surrounding area, and placed them in labeled containers.

All shell fragments we collected from our experimental boulders could be attributed to predation by fish and not by crabs. In our laboratory experiments we noted that pile perch preying on snails immediately spat out small broken pieces of shell body whorl. However, they swallowed the columella and later excreted it in a mucus-coated pellet onto the tank floor. All shell fragments found in our field experiment were small broken pieces, and no columellas were seen. We also did not see pelleted fragments of columellas, probably because fish retreated to the subtidal before defecating. This is distinct from shell fragments formed as a result of predation by crabs, which typically have either an intact columella with a spiral gouge up the body whorl or broken pieces of columella (Zipser & Vermeij 1978, Lawton & Hughes 1985, Behrens Yamada & Boulding 1996).

Upon returning to the laboratory we counted the number of replicate patches in which we found shell fragments. We also counted the percentage of live snails that we had deployed that were recovered. The data were analyzed with contingency table analysis and with ANOVA.

Contingency Table Analysis

The different dates of the experiment were categorized into three separate spring (or large) tide series: A, B, and C. Tide A refers to dives from June 28 to July 1, Tide B is from July 12 to July 14, and Tide C is from July 29 to August 3, 1999. We assumed predation by pile perch when we recovered shell fragments on or near the rocks where snails were deployed. First, tidal series A–C were compared with each other in a pairwise fashion to test for differences in the mean percentage of the deployed patches that experienced predation using Fisher's Exact Test. Tidal series B and C showed no differences in mean percentage predation per day; they were therefore lumped together. Differences in mean percentage predation between high- and low-density patches were then compared within the two new groups using Fisher's Exact Test.

ANOVA

We compared the mean percentage of snails we recovered in the high patches vs. the low patches across the 12 days using a factorial ANOVA model with date and treatment as the factors. We then used Fisher's Least Significant Difference test to determine which pairs of means were significantly different.

RESULTS

Laboratory Experiments

The four pile perch in the consumption experiment ate an average of 33.3 ± 6.3 snails per day (mean ± SE). The largest fish (total length = 330 mm) ate significantly more snails ($P < 0.006$) than one of the smallest fish (total length = 280 mm). We observed that when the *L. sitkana* were placed in the bottom of the tanks they eventually crawled up to or above the water line, which made them inaccessible to the fish. We therefore reanalyzed the data considering only the consumption rate of the snails found at or below the water line but got very similar patterns. The fish ate

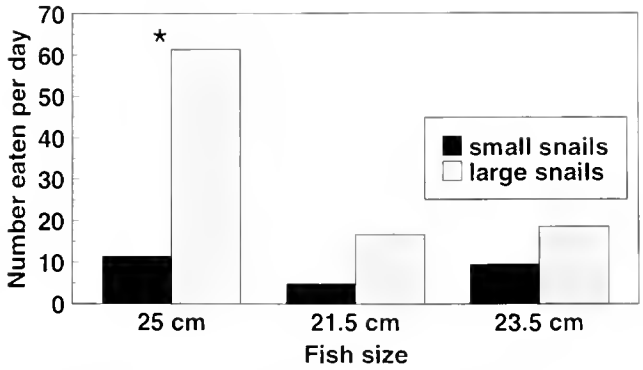

Figure 1. Size-selection for larger snails (*Littorina sitkana*) in the laboratory consumption experiment. *indicates a fish which ate significantly more large snails (Bonferroni pairwise comparisons, $P < 0.05$).

32–61% of the snails available with the largest fish again eating significantly more than the smallest fish ($P = 0.033$).

Only the largest of the three remaining pile perch (total length = 330 mm) in the size-selection experiment showed a significant preference for the extra-large size-class of *L. sitkana* over the smallest size-class (Fig. 1). There was a significant interaction between FISH and SIZE ($P < 0.001$) but no significant effect of DATE ($P = 0.319$).

The laboratory consumption experiment verified that the pile perch were very efficient predators on the snails, but only one fish would feed while being watched. One 250-mm fish required only 42.9 ± 8.02 sec (mean ± SE, $n = 19$) to consume each large *L. sitkana* (shell length 10 mm). The same fish became even more efficient at eating the snails by the next day and required only 19.2 ± 5.61 sec (mean ± SE; $n = 20$) to consume each snail.

Field Transects

The average density of pile perch in the intertidal at high tide was moderately low and was concentrated in the middle intertidal zone. In 1998, the average density of pile perch in the 0.8- and 1.1-m transects combined was an order of magnitude higher than the average density of striped perch (Table 1). In 1999, the densities of pile perch and striped perch in the 1.3-m transect were similar to those observed in the 0.8- and 1.1-m transects in the previous year (Table 1). However, in 1999 we also surveyed a 0.0-m transect where we observed no striped or pile perch (Table

TABLE 1.

Density of pile perch and striped perch in 1998 and 1999 in transects at Pebble Beach near Bamfield Marine Station.

Year	Species	Mean density	SE	n[d]
1998[a]	Pile perch	0.119/m^2	0.0205	20
	Striped perch	0.0205/m^2	0.0055	20
1999[b,c]	Pile perch	0.124/m^2	0.0221	16
	Striped perch	0.0275/m^2	0.00818	16

[a] Densities are taken from an average of 0.8 m and 1.1 m above 0.0 datum transects.

[b] Densities are taken from 1.3 m above 0.0 datum transect.

[c] No fish were seen at 0.0 m; $n = 6$.

[d] n is number of transects surveyed.

1). Most of the fish that we observed in both years were small. In 1998 the average length of the pile perch was 12.0 mm, and the average length of the striped perch was 11.2 mm (Table 2). In 1999, the average length was slightly larger for both species, with the pile perch averaging 15.2 mm and the striped perch averaging 13.5 mm (Table 2). In 1998 most fish were small, but some pile perch were more than 30 cm in length (Fig. 2). We found that experienced divers were good at estimating the total length of model fish. All three divers were able to place the models into the correct 5-cm size-class 95% of the time.

Our quadrat sampling of Pebble Beach estimated that the current densities of *L. sitkana* and other potential prey species for the pile perch were very low. The most common prey species found were the gastropods *L. scutulata* and *Tegula funebralis*, and even these were not abundant (Table 3). *L. sitkana* was especially rare, with only one found in the entire quadrat survey and only three more found after walking the entire beach. The density of mussels (*M. trossulus*) was very low, but the abundance of barnacles (*Balanus glandula*) was high.

Field Experiments

Contingency Table Analysis

A total of 40% of all the patches deployed in this experiment ($n = 70$) were found by fish within 50 min. We found that the number of patches where we found shell fragments (i.e., predation) was significantly higher in the second and third tidal series than the first (A vs. B: $P < 0.004$, df = 1, $n = 41$; A vs. C: $P < 0.036$, df = 1, $n = 52$). The percentage of patches in each tidal series where we found shell fragments is shown in Figure 3. The second and third tidal series were not significantly different from each other in the number of patches that experienced predation and were thus lumped together for analysis of treatment effects (B vs. C, $P = 0.37$, df = 1, $n = 47$). In the first tidal cycle, significantly more of the high-density treatments experienced predation (Table 4). In the second and third tidal series, there was no difference in the percentage of the patches that experienced predation between the high- and the low-density treatments (Table 4). The mean percentage predation in the low- and high-density treatments is shown in Figure 3 for each tidal series. In all patches where shell fragments were found, an average of 81 ± 0.06% ($n = 26$) of the snails deployed were eaten.

ANOVA

A significant DATE × TREATMENT interaction was found for the percentage of recovered snails (Table 5); therefore, we present only the interaction means (Fig. 4). We recovered a significantly lower proportion of snails from the high-density patches than from the low-density patches on 2 of the 12 days of the experiment (June 28 and July 13). We observed an inverse density-dependent trend on 3 days, but these were not significant (Fig. 4).

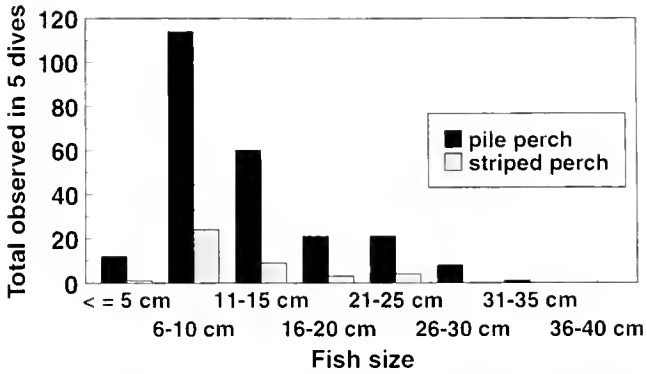

Figure 2. Size-distribution of the pile perch, *Rhacochilus vacca*, and the striped perch, *Embiotoca lateralis*, seen in intertidal transects in 1998.

The divers made some additional observations on foraging pile perch. In 1998, divers at Pebble Beach watched two 35-cm pile perch consume a patch of deployed snails and noted that they required only 9 min to consume a total of 17 snails. As previously mentioned, in 1999, divers left the patches for at least half an hour after they had deployed the snails. However, by the third spring tidal series they noticed that some fish began to circle the divers before all the snails were deployed. Although divers were not present during the entire time that the snail patches were deployed, any predation that was observed was always by pile perch.

DISCUSSION

This study shows that pile perch were the major consumers of the large *L. sitkana* we deployed in the Pebble Beach intertidal during diurnal high tides. Striped perch were much less common in the field transects at Pebble Beach, and we did not observe striped perch eating the snails we deployed. The pile perch has previously been implicated as an important predator of littorinid gastropods on wave-sheltered shores of the Northeastern Pacific (McCormack 1982, Boulding et al. 1999). However, there has been some question about its importance because at some sites predation rates by

TABLE 2.

Mean size of pile perch and striped perch in 1998 and 1999 in transects at Pebble Beach near Bamfield Marine Station.

Year	Species	Mean size (cm)	SE	*n*
1998[a]	Pile perch	12.01	1.35	237
	Striped perch	11.17	1.14	41
1999[b]	Pile perch	15.2	1.63	99
	Striped perch	13.5	3.06	22

[a] Lengths are taken from an average of 0.8 m and 1.1 m above 0.0 datum transects.
[b] Lengths are taken from 1.3 m above 0.0 datum transect.

TABLE 3.

Abundance and mean size (shell length) of mollusc prey present at Pebble Beach near Bamfield Marine Station[a]

Tidal height[b]	Prey species[c]	Mean density (m^{-2}) ± SE	Mean size (mm) ± SE	*n*
1.5 m	*Littorina scutulata*	10.0 ± 3.36	5.21 ± 6.44	50
	Tegula funebralis	1.6 ± 1.16	12.60 ± 1.20	8
2.2 m	*Littorina scutulata*	15.6 ± 4.09	5.77 ± 5.90	78
2.8 m	*Littorina scutulata*	8.4 ± 2.42	5.59 ± 1.96	42
3.2 m	*Littorina scutulata*	4.4 ± 1.80	6.09 ± 0.50	22

[a] A total of five quadrats were done per tidal height.
[b] Tidal height above 0.0 datum.
[c] Also one *Littorina sitkana* shell length 9.0 mm.

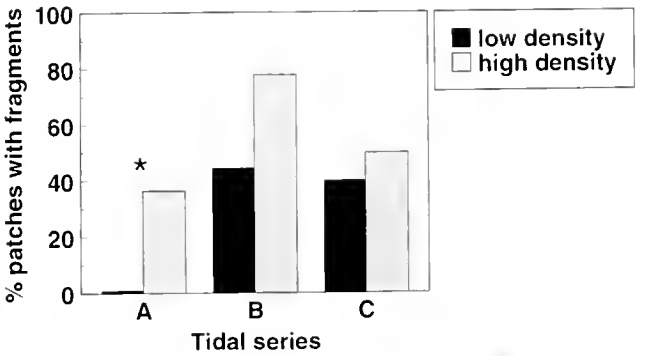

Figure 3. Percentage of snail patches deployed where shell fragments were found at Pebble Beach in high-density and low-density treatments for tidal series A (June 28 through July 1, 1999), B (July 12 through July 14, 1999), and C (July 28 through August 3, 1999). *Significant differences between densities (Fisher's Least Significant Difference test, $P < 0.05$).

pile perch have been estimated to be close to zero (Behrens Yamada & Boulding 1996). In our experiment the pile perch found 40% of the deployed patches of snails. Within the patches found by the fish, the consumption rate averaged 81%. This suggests that pile perch have the potential to severely limit the size of *L. sitkana* populations. We doubt that predation rates on the natural snail population on Pebble Beach are nearly so high because the population is currently at a very low density. However, natural populations of *L. sitkana* do occur at higher densities at other locations (McCormack 1982), and pile perch predation rates on these populations may be high.

Adult pile perch exhibited a very high foraging efficiency when feeding on large *L. sitkana*. The pile perch consumed snails at a very high rate (19.1 sec per snail). The largest fish showed a size preference for large snails, which makes it unlikely that certain size-classes of *L. sitkana* have a size refuge from pile perch. In addition, large pile perch were moderately abundant in the intertidal zone at high tide at Pebble Beach and reached fish densities of 0.119/m^2. This is an order of magnitude higher than the only previously reported densities for pile perch of 0.0039–0.0109/m^2 in kelp beds off southern California (Ebeling et al. 1980). These data suggest that the pile perch could be an important limiting factor for the population of *L. sitkana* at this site.

TABLE 4.

Number of high- and of low-density patches of *Littorina sitkana* deployed at Pebble Beach in 1999 that were recovered with and without shell fragments resulting from predation by pile perch.

Tide series	Patches with fragments	Patches without fragments	df	*P* value[d]
A,[a] High-density (50 snails)	0	12	1	
Low-density (5 snails)	4	7		0.037
B[b] & C[c], High-density (50 snails)	10	14	1	
Low-density (5 snails)	14	9		0.248

[a] Trials performed from June 28 through July 1, 1999.
[b] Trials performed from July 12 through July 14, 1999.
[c] Trials performed from July 28 through August 3, 1999.
[d] Probability values are from Fisher's Exact Test for particular tide series.

TABLE 5.

Analysis of Variance of percentage of snails recovered.[a] Factors are DATE (Date of Experiment) and DENSITY (Density of Snails).

Source	SS	df	MS	*F*	*P* value
DATE	8.469	11	0.770	3.827	0.001
DENSITY	0.760	1	0.760	3.777	0.058
DATE × DENSITY	4.833	11	0.439	2.184	0.032
Error	9.255	46	0.201	0.201	

[a] Percentage of live snails (*Littorina sitkana*) recovered after exposure to predation during one high tide period. Data were transformed with an angular transform before analysis.

When a pile perch found a patch of deployed snails in our field experiment, it ate the majority of the snails. Density-dependent predation can result from a predator finding high-density patches of prey more often or from the predator spending more time in high-density patches once they are found (Krebs & Davies 1991). Consumption rates were high in all patches that were found suggesting that the fish were not spending more time in high-density patches than in low-density patches. Therefore, any density-dependent predation we observed probably resulted from the fish finding high-density patches more easily than low-density patches. No work has been done specifically on the searching behavior of the pile perch, but they are known to be visual predators that feed during the day (Ellison et al. 1979). Many other benthivorous fishes that feed diurnally are know to use vision almost exclusively to find prey (Keenleyside 1979).

Our analysis by tidal series found significant density-dependent predation in the first tidal series (A) but not the second (B) and third (C). We doubt the lack of significance in tidal series B and C is due to low statistical power because sample sizes were smallest in tidal series A. Furthermore, we also observed a low frequency of density-dependent predation when we analyzed our experiment with ANOVA, detecting it on only 2 of the 12 days.

There may be several reasons why our study did not frequently detect density-dependant predation of *L. sitkana* by pile perch. In tidal series C, the divers noticed several large pile perch following them that consumed the snails as soon as they were deployed.

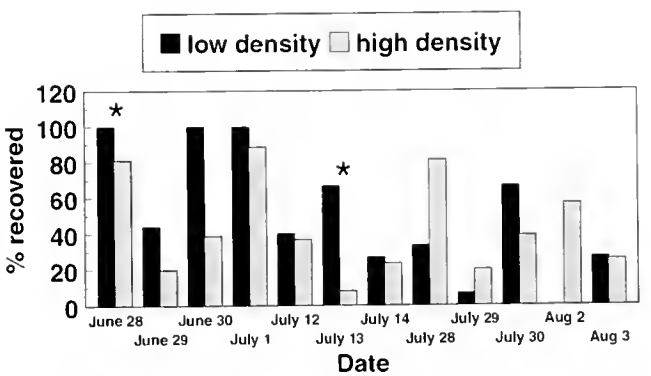

Figure 4. Mean percentage of snails (*Littorina sitkana*) recovered from the low- and high-density treatments on six different sampling dates. *Dates on which a significantly higher percentage of snails was recovered from the low-density treatment (Fisher's Least Significant Difference test, $P < 0.05$).

This suggests that the fish had become habituated to the divers and used their presence as a cue to find the patches of snails. This behavior may have made it difficult to detect density-dependent predation.

Another explanation for the low frequency of density-dependent predation is that the high consumption rate of snails by pile perch in both the high- and the low-density treatments made it difficult to detect subtle density-dependent predation. Our ability to detect density dependence was further reduced by our inability to distinguish snails that had been consumed from those that had simply migrated away from the top of the boulder. A single missing snail would affect the percentage recovered in the low-density treatment much more strongly than in the high-density treatment. For example, two snails migrating from the low density-treatment where a total of only five were deployed would result in a 60% recovery of snails, whereas two snails migrating from the high-density treatment would result in a 96% recovery.

In addition, the low-density treatments may have been in fact high density when compared with the present ambient density of possible prey species at this site. Almost no *L. sitkana* were found in our quadrats at Pebble Beach. Two other gastropod species were present at Pebble Beach, *L. scutulata* and *Tegula funebralis*, but neither are preferred prey of pile perch. Adult *Tegula* are rejected by pile perch (McCormack 1981), whereas the *L. scutulata* present on this beach averaged only 5.21 mm in shell length and are likely too small to be profitable. We suspect that the intense predation by pile perch at Pebble Beach keeps the densities of their preferred prey very low. Indeed, the low densities of potential prey items found there in 1999 cause us to question why the fish are venturing in the intertidal at all. The pile perch may be feeding on the barnacles there as was observed by our divers in 1998. Barnacles were reported to make up 56–75% of the stomach contents of large pile perch foraging on an artificial reef off the Washington coast (Hueckel & Stayton 1982).

Given the high predation rates we observed, we find it highly unlikely that any of the deployed snails would have survived if left out for another hour. The ambient density of prey may have been exceptionally low at Pebble Beach in 1999 that an unusually high proportion of the deployed snails were found and eaten. McCormack (1981) found much higher densities of *L. sitkana* at Pebble Beach in 1980 (10 snails/m^2 at 1.5 m tidal height and 970 snails/m^2 at 2.2 m in 1980) than we did (Table 3). We hypothesize that, rather than being regulated at an equilibrium density, there may be large fluctuations over time in the density of this *L. sitkana* population.

This study did not find predation by the pile perch on deployed patches of littorinid prey to be consistently density dependent. Consequently, we lack strong evidence that this predator has the potential to be an important regulating factor for the *L. sitkana* population at this site. Nevertheless, its high success rate at locating patches, its high foraging efficiency, and the high mortality rates we observed for the deployed prey suggests that the pile perch is an important limiting factor for the snail population at this site.

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RADULAR MYOGLOBIN AS A MOLECULAR MARKER IN LITTORINID SYSTEMATICS (CAENOGASTROPODA)

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ABSTRACT Radular myoglobin (Mb) was investigated in 288 specimens of 10 littorinid species using vertical polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF). Within the genus *Littorina* the two most basal species, *L. striata* and *L. keenae*, have Mb patterns that correspond to those of the genera *Littoraria* and *Nodilittorina*, while the sibling species *L. scutulata* and *L. plena* have identical Mb profiles that consistently differ from those of *L. littorea*, *L. saxatilis*, *L. compressa* and *L. arcana*. In contrast to previous claims, Mb does not consistently separate the sibling rough periwinkles *Littorina saxatilis* and *L. arcana*. These data suggest (1) that the *Nodilittorina/Littoraria* Mb profile in *L. striata* is not unique within the genus *Littorina* and therefore does not refute the assignment of *L. striata* to this genus, and (2) that *L. scutulata* and *L. plena* occupy a separate position compared to the other species of the subgenus *Littorina*. This latter result supports the suggestion that *L. scutulata* and *L. plena* may constitute a separate subgeneric taxon. Finally, the IEF Mb profiles of *Nodilittorina hawaiiensis* and *Cenchritis muricatus* were nearly identical to the *Nodilittorina/Littoraria* Mb pattern. Yet, PAGE of Mb in *Cenchritis muricatus* suggests a tentative Mendelian polymorphism. It is concluded that littorinid Mb may not be a useful marker to distinguish closely related species, but rather provides information on 'higher level' systematics.

KEY WORDS: Caenogastropoda, isoelectric focusing, Littorinidae, myoglobin, protein electrophoresis, systematics

INTRODUCTION

With only two published studies prior to 1998, radular myoglobin (Mb) has not been widely used in littorinid systematics and population genetics (Wium-Andersen 1970, Jones 1972). This is not unexpected since the genetic background of Mb variation in periwinkles remains obscure and controversial (Olabarria et al. 1998). Nevertheless, it has been shown that simple protein electrophoretic surveys of Mb can provide useful data for littorinid systematics and population genetic analyses (De Wolf et al. 1998, Medeiros et al. 1998, Olabarria et al. 1998). In this context, Medeiros et al. (1998) observed that within the genus *Littorina* there was considerable intra- and interspecific Mb differentiation. This allowed, for example, to separate the sibling rough periwinkles *Littorina (Neritrema) saxatilis* and *L. (N.) arcana*. In contrast, the Mb patterns of two *Littoraria* species and three *Nodilittorina* species were almost, if not completely, identical. Interestingly, the Mb profile of *L. striata* was similar to that of *Littoraria* spp. and *Nodilittorina* spp., but differed conspicuously from that of *Littorina* spp. This latter result could be interpreted in two ways: either *L. striata* is not a *Littorina* or the Mb profile of *L. striata* represents a plesiomorphic condition within *Littorina*.

The present contribution is a follow-up of the work by Medeiros et al. (1998). In particular, we will: (1) test the reliability of Mb as species marker to differentiate between *L. saxatilis* and *L. arcana*, (2) compare the Mb profile of *L. striata* with those of three other basal *Littorina* species, which are supposed to represent its closest relatives (*L. keenae*, *L. scutulata* and *L. plena*) (Reid 1990, Reid 1996, Reid et al. 1996), and (3) evaluate the electrophoretic Mb monomorphism in *Littoraria* and *Nodilittorina* (and related genera) by resolving Mb patterns in two additional species (*Nodilittorina hawaiiensis* and *Cenchritis muricatus*).

Throughout this article we will follow the taxonomy and nomenclature proposed by Reid (1989, 1996). We will use the abbreviation '*L.*' for the name *Littorina*, whereas the name *Littoraria* will be written in full.

MATERIALS AND METHODS

Electrophoretic profiles of radular Mb (and other structural proteins in the radular muscle) were surveyed in 288 periwinkles representing 10 species (Table 1). After collection specimens were transported alive or in liquid nitrogen to the laboratory, where they were stored at −80°C. Sample preparation was as described by Medeiros et al. (1998) and adapted in order to reduce possible artificial Mb variation caused by oxidative denaturation (e.g. Di Iorio 1981, Righetti 1983). Therefore individual radular tissue homogenates were prepared by thawing frozen snails, crushing their shells and dissecting the radular muscle in cold distilled water. The radular muscle was then blotted on filter paper and homogenized in a 0.1% KCN (w/v) in 20% (v/v) glycerol solution, in a ratio of 20µl solution per mg tissue. KCN converts Mb to cyanometmyoglobin, which is more stable and prevents denaturation to hemichromes (Atassi 1964, Di Iorio 1981). Crude homogenates were subsequently centrifuged for 30 min at 27200 × *g* (15000 r.p.m.) at 4°C. The resulting supernates were stored at −80°C until used for electrophoresis.

Vertical polyacrylamide gel electrophoresis (PAGE) was performed in 80 × 80 × 0.75 mm gels ('Mini Protean II' apparatus of Biorad) with a gel strength of 7% and using a discontinuous buffer system with Tris/HCl pH 9.0 as gel buffer and Tris/Glycine pH 9.0 as tray buffer (Backeljau 1989). Otherwise, PAGE procedures and conditions were as described by Medeiros et al. (1998). The protocols of these authors were also followed to perform horizontal isoelectric focusing (IEF) in pH gradients 3–9 and 4–6.5. PAGE and IEF gels were stained for general proteins (including Mb) with Coomassie Brilliant Blue and specifically for Mb with a benzidine recipe (Medeiros et al. 1998).

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TABLE 1.

List of littorinid species and collection sites screened for Mb variation

Species	Locality	*N*
Melarhaphe neritoides (Linnaeus, 1758)	Pico, Azores	10
Cenchritis muricatus (Linnaeus, 1758)	Isla Margarita, Venezuela	15
Nodilittorina hawaiiensis Rosewater & Kadolsky, 1981	Hawaii	15
Littorina (Littorina) striata King & Broderip, 1832	São Miguel, Azores	15
	Pico, Azores	15
	Terceira, Azores	15
	Madeira	4
Littorina (Planilittorina) keenae Rosewater, 1978	San Simeon, CA, USA	15
	Leo Carrillo Beach, CA, USA	15
	Morro Bay, CA, USA	15
Littorina (Littorina) plena Gould, 1849	Leo Carrillo Beach, CA, USA	15
	Morro Bay, CA, USA	15
Littorina (Littorina) scutulata Gould, 1849	Morro Bay, CA, USA	4
Littorina (Neritrema) compressa Jeffreys, 1865	Port Bhéal an Duin, Ireland	15
	Trébeurden, France	15
Littorina (Neritrema) arcana Hannaford Ellis, 1978	Ravenscar, UK	15
	Robin Hoods Bay, UK	15
Littorina (Neritrema) saxatilis (Olivi, 1792)	Venice, Italy (type loc.)	15
	Robin Hoods Bay, UK	15
	Ravenscar, UK	15
	São Miguel, Azores	15

RESULTS

All specimens of *L. saxatilis*, *L. arcana* and *L. compressa* had the same monomorphic Mb profile, both with PAGE (Fig. 1) and IEF (Fig. 2). These profiles corresponded with the *L. saxatilis* profile reported by Medeiros et al. (1998), while the alternative Mb profile, said to be typical of *L. arcana* (Medeiros et al. 1998: figs 2, 4), was not observed here.

Both PAGE (not shown) and IEF revealed that *L. scutulata* and *L. plena* have identical Mb profiles, which resemble that of *L. saxatilis*, except for the fact that with IEF the whole *L. saxatilis* Mb profile was slightly shifted toward a higher pH (Fig. 3). *L. keenae*, on the contrary, had a very different Mb profile which was shared with *L. striata*. Yet, with IEF *L. striata* revealed an additional Mb fraction at a pI of about 4.6, which was not observed in the other littorinids studied here (Fig 3). On the other hand, *L. striata* and *L. keenae* showed a more or less strong Mb band at a pI around 6.3. This band was lacking in the subgenera *Littorina* and *Neritrema*, but was present in *N. hawaiiensis*, *C. muricatus* and *M. neritoides*. For the remainder the IEF profiles of *N. hawaiiensis* and *C. muricatus* were similar to those of *L. keenae* and *L. striata*, although they did not show the Mb fraction at pI 4.6 of *L. striata* (Fig. 4). Finally, similar to the results of Medeiros et al. (1998), the Mb profile of *M. neritoides* was indistinguishable from that of *L. striata* with PAGE (not shown), but appeared to be distinct with IEF (Fig. 3).

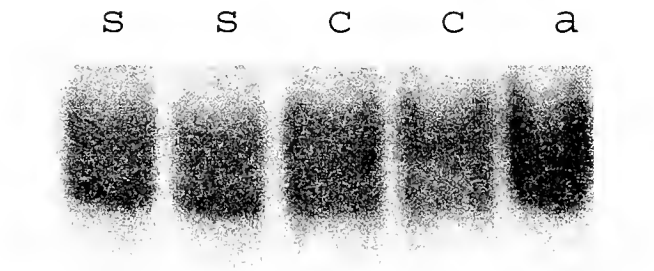

Figure 1. Benzidine staining of PAGE profiles of radular Mb in *L. arcana* (a), *L. compressa* (c) and *L. saxatilis* (s).

Surprisingly, in contrast to the apparent Mb monomorphism of *C. muricatus* revealed by IEF, PAGE of the same individuals yielded a tentative Mendelian polymorphism reminiscent of a monomeric protein coded by a single locus with two alleles (Fig. 5).

DISCUSSION

The present analyses show that the alleged species specific Mb differentiation between *L. saxatilis* and *L. arcana* (Medeiros et al. 1998) is not foolproof, since the *L. saxatilis* profile also occurs in *L. arcana*. Hence, as Medeiros et al. (1998) screened only one population of *L. arcana* (from Great Castle Head near Dale Fort, UK) and did not apply the KCN protocol to reduce artificial Mb variation, it seems worthwhile to screen a new batch of animals from this population in order to confirm the existence of two electrophoretic Mb types in *L. arcana*.

The fact that *L. scutulata* and *L. plena* have identical Mb patterns is not surprising, given the close relationship between both species (Mastro et al. 1982, Murray 1982, Reid 1996, Rugh 1997). It is however, interesting that the Mb profile of these two species differs from that of *L. saxatilis*, *L. arcana*, *L. compressa*, and *L. littorea* (this latter by inference from Medeiros et al. 1998), while

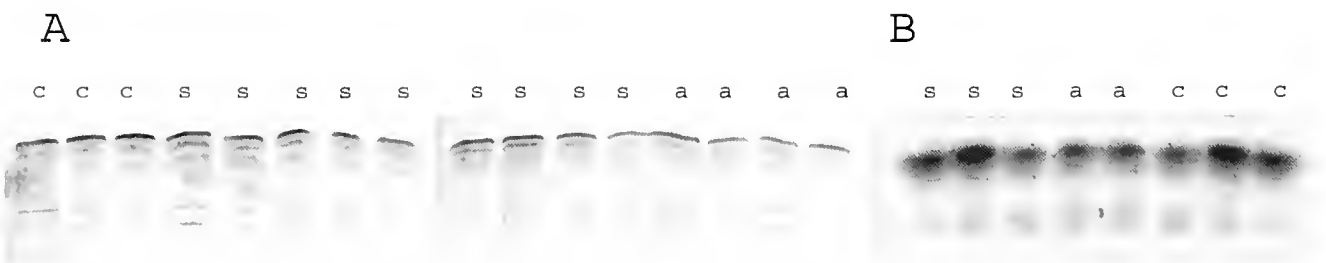

Figure 2. IEF profiles (pH 4–6.5) of radular Mb in *L. arcana* (a), *L. compressa* (c) and *L. saxatilis* (s), stained with Coomassie Brilliant Blue (A) and benzidine (B).

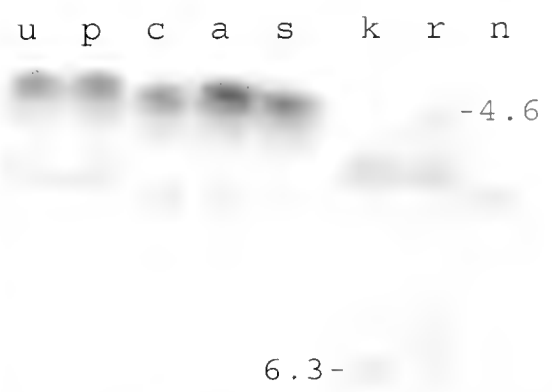

Figure 3. Benzidine staining of IEF profiles (pH 4-6.5) of radular Mb in *L. arcana* (a), *L. compressa* (c), *L. keenae* (k), *M. neritoides* (n), *L. plena* (p), *L. striata* (r), *L. saxatilis* (s), and *L. scutulata* (u). The arrow indicates the special band at pH 4.6 in *L. striata*; the triangle indicates the band at pH 6.3.

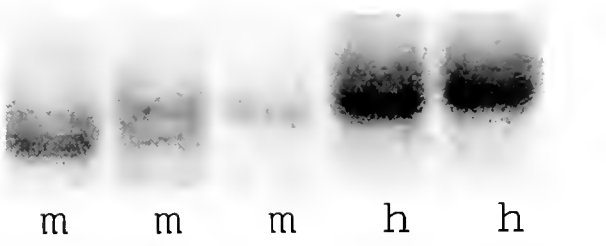

Figure 5. Benzidine staining of PAGE profiles of radular Mb in *C. muricatus* (m) and *N. hawaiiensis* (h). Note the suggestive Mendelian-like variation in *C. muricatus*.

the Mb profiles of *L. keenae* and *L. striata* differ even more conspicuously from the Mb profile of these four species. Instead the Mb profiles of *L. keenae* and *L. striata* are similar to those of *Nodilittorina* spp., *Littoraria* spp. and *Cenchritis* sp. reported here and by Medeiros et al. (1998). These data are consistent with current taxonomic practice to place *L. striata* and *L. keenae* in the separate monotypic subgenera *Liralittorina* and *Planilittorina* (Reid 1996), such that the Mb pattern of *L. keenae* could have been derived from that of *L. striata* by the loss of the supposedly autapomorphic Mb band at pI 4.6 in *L. striata*. The Mb profile of *L. scutulata* and *L. plena* would then represent a synapomorphy distinguishing both species from the other *Littorina* and *Neritrema* species, while Mb profiles of *L. littorea*, *L. saxatilis*, *L. compressa* and *L. arcana* would unite the subgenera *Littorina* and *Neritrema*. Finally, the Mb patterns reported by Medeiros et al. (1998) for *L. arcana* (if correct), *L. fabalis* and *L. obtusata* may involve still further derived states. Given that in the consensus phylogeny of the genus *Littorina* (Reid 1996: Fig. 119) *L. scutulata* and *L. plena* form an independent clade (making the subgenus *Littorina* paraphyletic), our Mb data support the suggestion that these latter two species may constitute a separate subgeneric group.

Although this scenario fits the currently accepted phylogeny of the genus *Littorina*, the Mb patterns are also compatible with the alternative idea that *L. striata* may be not a *Littorina*. Yet, in that case *L. keenae*, whose assignment to *Littorina* has never been challenged, would become the most basal branch of the genus, suggesting that a *Nodilittorina/Littoraria*-like Mb profile (like in *L. striata* and *L. keenae*) is not a priori inconsistent with the genus *Littorina*. However, since electrophoretic mobilities are not reliable to infer homology and/or identity, the present Mb data are essentially phenetic. Hence apparent similarities may not necessarily indicate common descent, particularly not in a molecule like Mb which may be subject to functional constraints that may cause homoplasy. Nevertheless, the Mb data do suggest that a sequence analysis at the amino acid and nucleotide level could help to understand littorinid phylogeny and functional ecology. Such studies have been done for abalones (Suzuki et al. 1997) and anaspids (Rinaldi & Ophir 1998)

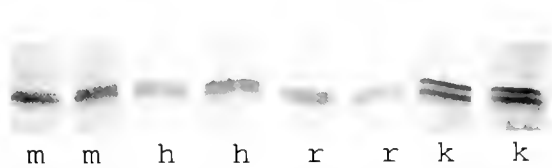

Figure 4. Coomassie Brilliant Blue staining of IEF profiles (pH 4-6.5) of *N. hawaiiensis* (h), *L. keenae* (k), *C. muricatus* (m), and *L. striata* (r).

Finally, the IEF Mb data on *N. hawaiiensis* and *C. muricatus*, add to the Mb monomorphism in the genera *Littoraria* and *Nodilittorina* (Medeiros et al. 1998) and extend this observation to the genus *Cenchritis*. However, they contradict the alleged species specific Mb variation in *Littoraria* and *Nodilittorina* as reported by Jones (1972), who used PAGE. Probably this discrepancy is due to technical issues and/or hidden Mb heterogeneity. Indeed, when we applied PAGE in *C. muricatus*, we also detected variation that was not uncovered by IEF. Hence, combined with the observations of Medeiros et al. (1998) on the hidden PAGE differentiation in *M. neritoides* that was resolved by IEF, it is obvious that PAGE and IEF are complementary in the analysis of littorinid Mb variation. However, whether the *C. muricatus* patterns really involve a Mendelian polymorphism, needs further scrutiny in view of the confusing evidence on the genetic background of littorinid Mb (Olabarria et al. 1998).

In conclusion, the present data confirm Medeiros et al.'s (1998) claim that littorinid Mb are useful systematic markers that can provide the same kind of information as do the haemoglobins in freshwater snails (Bailey et al. 1986) and fishes (e.g. Basaglia & Callegarini 1987, Macaranas et al. 1996, Rizzotti & Gioppato 1999), or the haemocyanins in terrestrial gastropods (e.g. Symondsen & Walton 1994) and crustaceans (e.g. Mangum 1996, Mangum & McKenney 1996). Nevertheless, littorinid Mb are less suited to differentiate closely related species, but on the other hand seem quite informative for higher level systematics. Unfortunately, the present knowledge on littorinid Mb is still far too scanty to fully exploit them for ecophysiological, population genetic, taxonomic and phylogenetic investigations.

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HERITABILITY OF SHELL TRAITS IN WILD *LITTORINA SAXATILIS* POPULATIONS: RESULTS ACROSS A HYBRID ZONE

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ABSTRACT There is a hybrid zone in *Littorina saxatilis* from exposed Galician rocky shores (northwest Spain) where two ecotypes, ridged and banded and smooth and unbanded are found to be associated with distinct habitats and shore levels. On the mid-shore, however, the two ecotypes and a variable percentage of hybrids can be found in sympatry. Furthermore, many shell traits present a clinal distribution along the vertical environmental gradient within ecotype, although very little knowledge about its genetic basis is available. This species is viviparous, and every female has a brood pouch containing many embryos. This makes it possible to use the shelled embryos from the brood pouch of every female to estimate the heritability of morphological traits (by full-sib correlation and offspring–mother regression). We took seven samples of 20 females across two vertical transects and found significant heritabilities for most shell traits and a multivariable factor score. The average narrow-sense heritability was 0.102, and the average broad-sense heritability was 0.489, although the present estimates can be somewhat biased. The high correlation observed between the genotypic variance (estimated by full-sibs correlation) and the additive variance (estimated by offspring–mother regression) as well as the similar results obtained after transforming the variables correcting for scale effects support the existence of a common additive genetic component in both kinds of heritability. Genetic variability significantly differed between shore levels. Interestingly, hybrids did not show an increase in genetic variability with respect to both pure ecotypes, which perhaps suggests that natural selection is eroding their variability. These results complement a previous claim about the adaptive shell polymorphism in Galician *L. saxatilis* populations

KEY WORDS: natural selection, hybridization, clinal variation, genetic variability, shell polymorphism, embryo shell

INTRODUCTION

Littorina saxatilis (Olivi) is a polymorphic species from North Atlantic intertidal shores (Fretter & Graham 1980). The species inhabits a wide range of different ecological niches, from estuaries to rocky shores or even salt marshes (Reid 1996). Coupled with these habitat differences there is a known morphological and behavioral population differentiation, which has been traditionally interpreted an as example of adaptive polymorphism (Janson 1983, Reid 1993, reviewed in Reid 1996). However, to be adaptive, a polymorphism must be first heritable (Endler 1986), which is usually a priori assumed in natural populations without further experimental evidence. A few studies have tried to estimate the proportion of genetic vs. phenotypic variability by rearing families in the laboratory (Johannesson & Johannesson 1996, Ward et al. 1986, Ward et al. 1991, Warwick 1983, Warwick et al. 1990), but this approach is troublesome, very time consuming, and, for some ecotypes (sensu Turesson 1922), very difficult (see Warwick et al. 1990). Yet, Newkirk and Doyle (1975) have suggested applying classical quantitative genetics methods to estimate genetic variances for quantitative traits in wild populations of this and other species with similar life-history characteristics. Because *L. saxatilis* females usually carry a brood pouch with many embryos (Fretter & Graham 1980, Reid 1996), pregnant wild females can be used as different families of full sibs, from which full-sib correlation and offspring–mother regression allow us to estimate the genetic variances for different shell traits (Falconer & Mackay 1996). Newkirk and Doyle (1975) found significant genetic differentiation between *L. saxatilis* populations allocated along an estuarine gradient. They found the lower genetic variability in those traits and population more affected by natural selection. They suggested that their polymorphism was maintained by natural selection.

On exposed Galician rocky shores, two sympatric ecotypes can be found associated with two different habitats (Johannesson et al. 1993): The ridged and banded form (RB) is usually found on upper shores among barnacles, whereas the smooth and unbanded form (SU) is typically found on lower shores among mussels. On the mid-shore, both barnacles and mussels overlap forming a patchy habitat in which both pure forms and some (5–40%) phenotypically intermediate forms (HY) can be found even copulating together. Every ecotype (RB, HY, and SU) partially aggregates in the mid-shore, showing particular preferences for different microhabitats (Otero-Schmitt et al. 1997). In fact, they do not mate randomly on the mid-shore, showing a noticeable but still incomplete assortative mating among ecotypes (Johannesson et al. 1995). The two ecotypes differ for many morphological, physiological, and behavioral traits (Erlandsson et al. 1998, Johannesson et al. 1993, Johannesson et al. 1997, Rolán-Alvarez et al. 1996, Rolán-Alvarez et al. 1997). Particularly, they show striking differences in shell size, morphology, and color polymorphism. Besides, this sharp ecotype distribution (an ecotone sensu Endler 1986), a more typical clinal distribution (sensu Endler 1986) across the shore gradient, can be found for many morphological traits within an ecotype (Cruz 2000). A genetic basis behind the presence of shell bands and ridges has been shown because females of each morph produce progeny of the same characteristics under laboratory conditions (Johannesson et al. 1993). However, there is no such proof for other quantitative traits or for quantitative estimates of the relative importance of genetic variability behind other shell differences. This information is fundamental if we want to understand fully the evolutionary forces that have modeled the observed ecotone and clines in shell traits along the environmental gradient.

We used the Newkirk and Doyle (1975) approach to estimate the heritability for different shell traits in wild populations of the Galician hybrid zone. We analyzed replicated transects along the vertical gradient and compared the results by full-sib correlation and mother–offspring regression. Because we are studying the

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trends in genetic variability on a zone where hybridization occurs (Johannesson et al. 1993, Rolán-Alvarez et al. 1996), we would expect that hybrids from mid-shore present some increase of their genetic variance due to hybridization for those traits being genetically differentiated between upper and lower shore populations. This latter null hypothesis assumes that the hybrid populations are stable and fertile (Johannesson et al. 1995). However, the results seem to contradict this expectation, suggesting that natural selection may be eroding the genetic variability in the hybrids or, alternatively, that the assumptions of such a model are false.

MATERIALS AND METHODS

Sampling

Adult *L. saxatilis* were sampled in June 1998 from two different localities, Centinela and Senín (separated by 3 km). They were picked along two vertical transects in intertidal rocky shore areas and five different samples (quadrats) within the transect (Fig. 1). Upper shore and upper mid-shore samples and lower shore and lower mid-shore samples covered the whole range of pure RB and SU populations, respectively. Upper mid- and lower mid-shore samples were closer to mid-shore samples (1–3 m) than to their respective upper or lower shore samples (4–10 m). In each sample, 100 snails (larger than 3 mm) within a 30–50 cm diameter circle were obtained and stored at −70°C. Later, they were randomly chosen from the sample, dissected, and sexed. The first 20 females with embryos within their brood pouch were used for the morphological study. The mid-shore sample included similar frequencies of RB, SU, and HY individuals. However, due to the low frequency of natural hybrids, it was not possible to obtain 20 pregnant HY females per sample (see Fig. 1). To avoid an unbalanced experimental design, hybrid data were excluded from some analyses (see below). Finally, 120 females (families) and 360 embryos of RB, 120 females and 360 embryos of SU, and 24 females and 72 embryos of HY were employed.

Morphological Variables

The shell of the female and the three largest embryos within their brood pouch were placed in an apertural top view and digitized to make several measurements (Fig. 2): shell height (SM1), aperture height (SM3), aperture width (SM4), and width of first spire whorl (SM7) following Cruz, Carballo and Rolán-Alvarez (submitted); distance from the center of the first whorl to the apex (Y) and ratio of the first whorl (R) following Newkirk and Doyle (1975); and top shell diameter (SD) and top aperture width (AW) following Rolán-Alvarez et al. (1996). Adult RB individuals were measured with an error of 0.0096 mm (eight magnifications required), adult SU individuals were measured with an error of 0.0042 (12.5 magnifications required), and both RB and SU embryos were measured with an error of 0.0016 mm (80 magnifications required) by a Leica MZ12 stereoscopic microscope. We statistically analyzed the parameter T (Y/R) and S (SM4/SM3), the latter with small modifications from Newkirk and Doyle (1975). We also used the distance from the end of first whorl to the apex (SM15) and the parallel to the maximum aperture length (SM16). Genetic analyses were accomplished on individual variables but also on a multivariable factor score obtained by principal component analysis. Classical statistical analyses and multivariable methods were done by the SPSS/PC package (version 9.0.1).

Genetic Analysis

The individual variables from embryo data were analyzed with a two-way nested analysis of variance (ANOVA) including the factor *shore level* (fixed; upper, upper-mid, lower-mid, and lower), the factor *locality* (random; Senín and Centinela), and the factor *family* (random; nested within the shore level × locality interac-

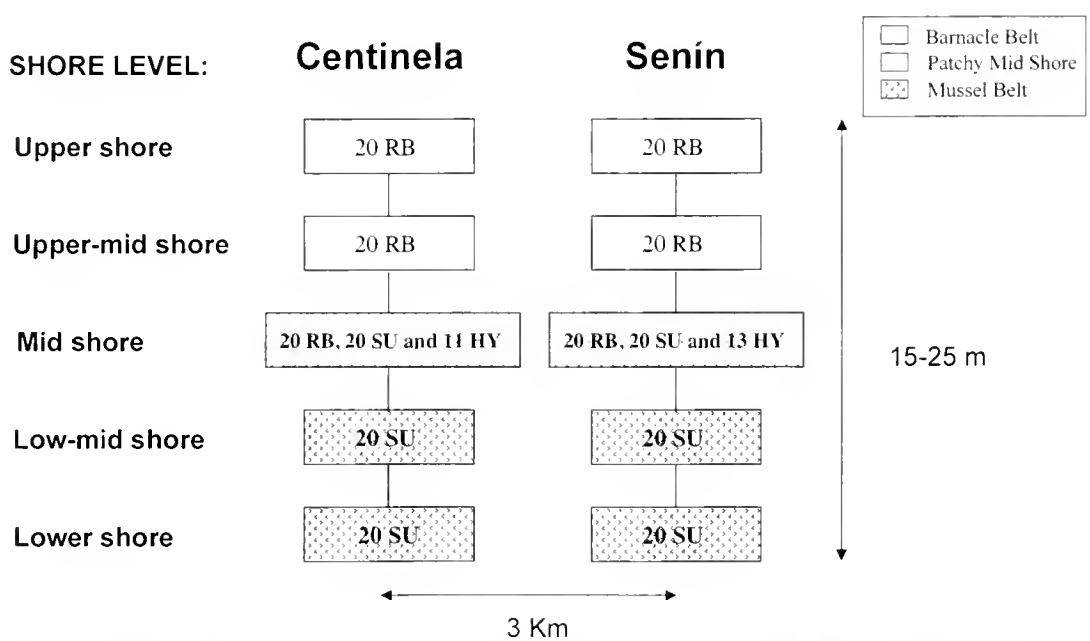

Figure 1. Sampling design. Every sample (quadrat) represents 20 adult females and the three largest embryos within their brood pouch, except in mid-shore samples where we tried to obtain 20 ridged and banded (RB), smooth and unbanded (SU), and phenotypically intermediate (HY) specimens. Upper shore samples were obtained on the barnacle belt, and lower shore samples were obtained on the mussel belt. Mid-shore samples where obtained in the patchy barnacle/mussel zone.

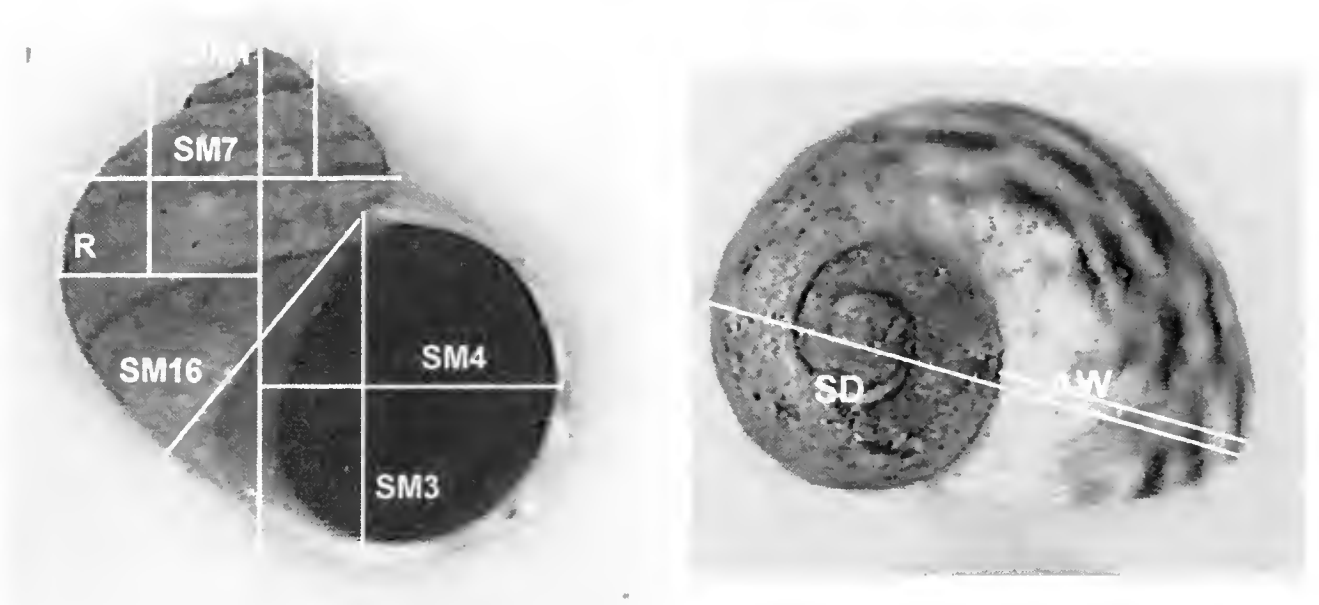

Figure 2. The morphological measurements studied on a smooth and unbanded (SU) individual. SM1, shell height; SM3, aperture height; SM4, aperture width; SM7, width of the first spire whorl; SM15, distance from the end of first whorl to the apex; SM16, the parallel to the maximum aperture length; R and Y following Newkirk and Doyle (1975); SD, top shell diameter; AW, top aperture width.

tion) on upper and lower shore samples. A second nested ANOVA was performed on mid-shore samples with the factor *morph* (fixed; RB and SU), the factor *locality* (random; Senin and Centinela), and the factor *family* (random; nested within the *morph* × *locality* interaction).

We assumed that embryos from the same brood pouch were true full sibs following Newkirk and Doyle (1975). These authors analyzed 12 independent groups of brood pouch embryos for an esterase polymorphism (with four alleles) and found that the embryos were full sibs in all cases except one (but see Newkirk & Doyle 1979). This assumption, if false, implies an underestimation of the heritability (it could be up to twice the observed amount). To minimize the risk of heterogeneity in progeny we used the three largest embryos (similarly sized) in every female. Classical genetic analyses of full sibs (ANOVA model 1) and offspring–mother regression were done following Falconer and Mackay (1996). The covariance between full-sib is one half of the additive variance plus one quarter of the dominance variance plus the variance due to the common environment, whereas the covariance between offspring and mother is exclusively one half of the additive variance. The former relationship can be used to obtain an estimate of the broad-sense heritability, whereas the latter allows us to obtain an estimate of the narrow-sense heritability. However, complex interactions and maternal effects may somewhat bias these estimates. For example, the phenotypic value of the mother for the character in question can directly influence the same character in the offspring. This effect can produce biases simultaneously in both full-sib correlation and offspring–mother regression. Another possible source of bias is the genotype–environment interaction, due to some environments causing intrinsically larger genotypic variability than others.

Snails from different shore levels usually have different average sizes (Johannesson et al. 1995). We have accomplished different statistical corrections to minimize this scale effect. First, we have preferentially used coefficients of variation rather than the heritability to compare results from different methods and samples (Sokal & Rohlf 1995, Houle 1992, Falconer & Mackay 1996). Furthermore, we have incorporated a transformation that corrects the scale effects between samples, using the linear regression of the mean against the standard deviation in every sample as suggested by Falconer and Mackay (1996). The transformation is $X = \log (x + a/b)$, where X is the transformed variable, x is the original variable, and a and b are the intercept and the slope of the linear regression of the mean on its standard deviation.

The comparison between mother and offspring measurements may be biased by other causes because the analyses assume that the two sets of data are obtained in individuals of the same age and for the same trait. The former is not true in our case, causing at best a scale effect (allometry) between mother and offspring. To avoid this, we repeated the analyses using the transformation suggested by Lynch and Walsh (1998) to correct for allometry with respect to size. The transformation uses the algorithm $Y = a^*X^b$, where a and b are the intercept and the slope, respectively, of the regression of the trait with the shell height. The residual of such a regression for every data point can be used to study the variation of the trait corrected for allometric effects (Packard & Boardman 1987).

RESULTS

The two-way nested ANOVA for individual variables presented in Table 1 show that the factor *shore level* was significant for most traits, whereas the factors *locality* and the *interaction* were significant only in a few cases. The factor *family* was significant in all cases, which may perhaps be due to genetic effects (see also below). In most cases we tried to correct for heteroscedasticity by using the square root or logarithmic transformations, succeeding for SM1, SM4, SD, and S. Therefore, for other traits, type I error must be taken with caution. Similarly, we found differences in the factors *morph* and *interaction* for most traits in mid-shore samples. Again we used square root and logarithmic transformation to correct for heteroscedasticity, being unsuccessful only with S. Similar results were obtained with the transformation

TABLE 1.

ANOVA on embryo measurements for upper/lower and mid-shore data sets.

	SM1	SM3	SM4	SM7	SM15	SM16	SD	AW	T	S
Upper/lower Shore										
Shore level	6.78***	30.30***	23.31***	4.39**	24.80***	0.05	11.40***	59.85***	7.99***	4.28**
Locality	1.18	0.34	18.59***	0.89	0.78	1.83	0.43	0.04	76.05***	80.68***
Interaction	1.42	1.59	1.31	2.08	6.46***	1.72	0.43	2.81*	9.61***	12.75***
Family (interaction)	3.94***	4.95***	3.04***	4.24***	2.15***	3.19***	5.89***	3.49***	1.59***	1.52**
Mid-shore										
Morph	13.54***	38.13***	30.96***	0.00	13.16**	5.96*	20.79***	70.64***	13.37	5.72*
Locality	3.50	0.81	6.08*	4.35*	18.28***	0.78	2.57	2.56	1.38	20.52***
Interaction	3.05	2.05	1.05	8.42**	4.71*	5.69*	5.03*	1.95	0.14	2.19
Family (interaction)	5.16***	4.88***	5.02***	4.37***	2.7***	3.94***	5.96***	1.80***	1.04	0.95

Abbreviations: SM1 = shell height; SM3 = aperture height; SM4 = aperture width; SM7 = width of the first spire whorl; SM15 = distance from the end of the first whorl to the apex; SM16 = the parallel to the maximum aperture length; SD = top shell diameter; AW = top aperture width; T = parameter (Y/R); S = SM4/SM3.

In the upper/lower data set the fixed factor is *shore level* (upper, upper-mid, lower-mid, and lower shore samples), whereas in the mid-shore data set the fixed factor is *morph* (ridged and banded vs. smooth and unbanded). In both cases the ANOVA incorporate the random factor *locality* (Senín and Centinela), the corresponding interaction, and the nested factor *family* (within the interaction). The analyses used 20 families per sample and three embryos per family. Hybrid families were excluded to avoid unbalanced designs.

of Falconer and Mackay (1996) (correcting for scale effects among samples) as well as traits divided by shell height (not shown). This suggests extensive morphological differences across shore levels and morphs, as well as among families, for most shell traits.

The genetic coefficient of variation and the heritability are presented for the measured variables across the environmental gradient in Table 2 for both full-sib and offspring–mother regression methods. The averages for the two localities are presented, with the significant cases designated by lowercase letters (with significant cases determined after sequential Bonferroni corrections underlined) in Senín (a) and Centinela (b), respectively. Furthermore, estimated heritabilities were much larger in full-sib analyses (broad-sense heritability) than in offspring–mother analyses (narrow-sense heritability). This may be caused by some contribution of maternal plus dominance effects to the full-sib resemblance but not to the offspring–mother resemblance. A more useful statistic to compare genetic variances between samples and methods may be the coefficient of variation, which partially corrects for scale effects. Interestingly, the coefficient of genetic variance (in full sibs) and the coefficient of additive variance (in mother–offspring regression) were highly correlated for most shell traits across shore levels (Table 2), suggesting a common component (probably the additive) in both kinds of estimates. Besides, the estimates seem to fall into two categories, upper or upper mid-shore samples (typical RB samples from upper shore), with very low coefficients of genetic variation and samples from mid- to lower shore with larger coefficients of genetic variation (Table 2). Scale effects may not cause these differences because those populations with the smallest variances had the greatest means for all measured variables. On the mid-shore another trend emerged: The coefficient of genetic variation of hybrids is never clearly larger than the corresponding estimates for both RB and SU ecotypes (Table 2). The values for hybrids are usually smaller or intermediate between pure ecotypes, which is exactly the opposite of that we would expect under a null hypothesis of hybridization for neutral genetic variability. The transformation of Falconer and Mackay (1996) for full sibs and the transformation of Lynch and Walsh for offspring–mother regression, which corrected for scale effects among samples and for embryo-adult allometry, respectively, rendered similar results in all variables: significant heritabilities for some traits and the same two trends mentioned above.

We could successfully reduce all the variables by principal component analysis of females. The first principal component (PC1) represented 79.9% of the overall variability. The relationship between the two main components (PC1 and PC2) is represented in Figure 3 for all females included in the analyses. The PC1 acts like a discriminant function between RB and SU morphs, having a clear biological meaning (a summary variable for all the morph differences). Figure 4 shows the coefficient of genetic variance (estimated from full-sib correlation) and the coefficient of additive variance (estimated from offspring–mother regression) for PC1 across shore levels. The estimates across sampling points were highly correlated ($n = 14$; $r = 0.832$, $P = 0.000$), which suggests that there is an important component of the covariance between relatives (probably the additive) in common (Fig. 4). Furthermore, this relationship was maintained when we used the Falconer and Mackay (1996) transformation to correct for scale effects among samples ($n = 14$; $r = 0.863$, $P = 0.000$). This supports the differences between shore levels in genetic/additive variances, although at present we cannot exclude some minor bias due to genotype–environment interaction or maternal effects. Again, the genetic variance estimates by full-sib correlation were larger than the additive variances estimated by offspring–mother regression, suggesting that maternal or dominance effects significantly contribute to the resemblance between relatives in the former. Interestingly, we observe the same two trends discussed above (Fig. 4) that upper and upper-mid populations presented significantly smaller genetic variances and that hybrids did not show an increase of genetic variances as would be expected under neutral hybridization.

DISCUSSION

Many studies have reported morphological, behavioral, and physiological polymorphism in littorinids (reviewed in Reid 1996). In some cases, laboratory experimentation, computer simulations, and natural studies have shown that natural selection may account for some of this polymorphism (Strushaker 1968, Janson

TABLE 2.

The coefficient of genetic variation and the broad-sense heritability (in parentheses) by full-sib correlation and the coefficient of additive variation and the narrow-sense heritability (in parentheses) by offspring–mother regression for different traits across the environmental gradient (shore level).

Analysis	Level	Morph	SM1	SM3	SM4	SM7	SM15
Offspring–mother regression	Upper	RB	9.06 (−0.004)	6.20 (0.000)	5.74 (−0.011)	7.90 (0.001)	26.61 (−0.003)
	Upper-mid	RB	6.15 a (0.019)	5.69 a (0.015)	5.91 (0.014)	7.53 a (0.031)	23.90 (0.016)
	Mid	RB	11.78 (0.034)	11.00 (0.020)	10.67 (0.023)	13.91 (0.067)	32.82 (0.004)
	Mid	HY	11.06 (0.028)	10.34 (0.011)	9.84 (−0.006)	12.23 (0.064)	27.67 b (0.055)
	Mid	SU	11.22 a (0.066)	10.03 a (0.065)	9.66 (0.058)	14.97 a (0.107)	38.64 (0.024)
	Lower-mid	SU	13.19 a b (0.122)	12.02 a b (0.138)	11.11 a b (0.118)	15.89 a b (0.155)	35.42 (0.040)
	Lower	SU	9.88 a (0.063)	9.43 a (0.077)	10.51 (0.023)	13.36 (0.075)	39.21 (0.029)
Full-sib correlation	Upper	RB	12.46 a b (0.308)	8.02 a b (0.382)	7.47 a b (0.398)	10.02 a b (0.422)	37.57 a (0.244)
	Upper-mid	RB	8.01 a b (0.374)	7.43 a b (0.370)	7.53 a b (0.411)	10.38 b (0.272)	34.99 b (0.191)
	Mid	RB	13.19 a b (0.666)	12.40 a b (0.650)	11.94 a b (0.667)	16.62 a b (0.526)	41.92 a b (0.407)
	Mid	HY	12.54 a b (0.625)	12.11 a b (0.555)	11.08 a b (0.639)	15.39 a b (0.438)	41.33 b (0.224)
	Mid	SU	13.59 a b (0.503)	12.15 a b (0.480)	11.81 a b (0.481)	17.99 a b (0.513)	52.76 a b (0.300)
	Lower-mid	SU	14.90 a b (0.642)	13.51 a b (0.647)	12.51 a b (0.644)	18.29 a b (0.582)	49.74 b (0.241)
	Lower	SU	12.30 a (0.401)	11.50 a (0.436)	13.27 a (0.190)	16.14 a b (0.509)	49.83 a b (0.416)
Correlation between methods			0.960**	0.992**	0.928**	0.990**	0.888**

TABLE 2.

Continued.

Analysis	SM16	Y	R	SD	AW	T	S
Offspring–mother regression	6.42 (0.006)	9.38 b (0.017)	7.91 a b (0.040)	5.07 (0.006)	6.71 (−0.008)	6.27 (0.160)	3.86 (0.154)
	6.46 (0.015)	8.61 (0.012)	6.46 a (0.031)	5.32 a (0.027)	7.80 (0.018)	7.16 (0.105)	3.17 (0.223)
	11.14 b (0.054)	12.44 (0.013)	18.46 b (0.072)	9.95 (0.039)	9.28 (0.020)	7.80 (0.156)	3.46 (−0.152)
	8.43 (0.019)	7.64 (0.042)	20.66 (0.072)	8.88 (0.020)	9.15 (−0.011)	7.58 (0.219)	3.44 (0.046)
	11.18 (0.036)	7.44 (0.024)	22.29 b (0.088)	9.69 (0.062)	8.70 (0.039)	5.85 (0.041)	3.38 (−0.240)
	12.42 b (0.100)	16.52 a b (0.085)	13.63 a b (0.140)	11.12 a b (0.118)	11.13 b (0.061)	8.94 (−0.173)	4.89 (0.093)
	8.04 b (0.060)	12.82 (0.068)	11.04 a b (0.126)	8.74 a (0.078)	10.94 a (0.072)	9.23 (0.195)	8.35 a b (0.040)
Full-sib correlation	9.05 b (0.251)	13.40 a b (0.229)	10.60 a b (0.327)	6.48 a b (0.405)	10.75 (0.071)	10.25 (0.089)	5.83 a (0.175)
	9.16 a b (0.240)	13.68 (0.088)	10.19 (0.101)	6.50 a b (0.482)	11.09 a (0.234)	11.20 (0.124)	4.99 (0.119)
	13.66 a b (0.476)	17.44 a b (0.446)	17.35 a b (0.572)	11.03 a b (0.684)	12.92 a (0.357)	12.36 a (0.097)	6.46 (−0.054)
	11.54 b (0.296)	13.31 a b (0.441)	17.45 a b (0.549)	9.91 a b (0.661)	12.17 b (0.327)	11.59 b (0.186)	6.24 (−0.022)
	13.61 a b (0.487)	14.76 a b (0.391)	17.62 a b (0.518)	11.73 a b (0.568)	13.58 b (0.167)	11.30 (−0.089)	6.07 (−0.061)
	14.48 a b (0.565)	21.31 a b (0.390)	15.81 a b (0.595)	12.16 a b (0.717)	13.46 a b (0.521)	13.16 a (0.201)	6.91 a (0.241)
	10.89 a (0.295)	18.44 (0.219)	13.39 a b (0.488)	10.46 a (0.447)	13.07 a (0.460)	13.48 b (0.187)	10.27 (0.148)
Correlation between methods	0.984**	0.792**	0.818**	0.989**	0.932**	0.730**	0.672**

In every sample we measured 20 adult females and the three largest embryos per female. The estimates are the mean across localities. The letter "a" represents significant heritability estimates, by analysis of variance (ANOVA) for Centinela samples ($P \leq 0.05$), and the letter "b" represents significant genetic variability estimates (by ANOVA) for Senin samples ($P \leq 0.05$). The F tests differed between full sibs ($df_1 = 19$; $df_2 = 40$) and offspring-mother ($df_1 = 1$; $df_2 = 18$) data sets (see also MATERIALS AND METHODS). Significant cases after sequential Bonferroni multitest corrections for the 14 different estimates within trait (Rice 1989) are underlined. **Significant Pearson correlation ($p < 0.01$) between the coefficient of genetic variance (in full sibs) and the coefficient of additive variance (in mother-offspring regression).

Abbreviations: SM1 = shell height; SM3 = aperture height; SM4 = aperture width; SM7 = width of the first spire whorl; SM15 = distance from the end of the first whorl to the apex; SM16 = the parallel to the maximum aperture length; Y = distance from the center of the whorl to the apex; R = ratio of the first whorl; SD = top shell diameters; AW = top aperture width; T = parameter (Y/R); S = SM4/SM3.

1983, Boulding 1990, Rolán-Alvarez et al. 1997), but only in a few cases is there data showing a genetic basis for the studied traits (Strushaker 1968, Murray & Clark 1966, Reimchen 1979, Boulding & Hay 1993, Johannesson & Johannesson 1996). The low frequency of studies showing genetic variability for the phenotypic polymorphism in the wild is frustrating because it is a serious drawback of any adaptive hypothesis. Besides, there is a theoretical good chance to be successful (nearly all examined quantitative traits have shown genetic variation; Falconer & Mackay 1996). Here, we have attempted quantitative genetic estimates (heritabili-

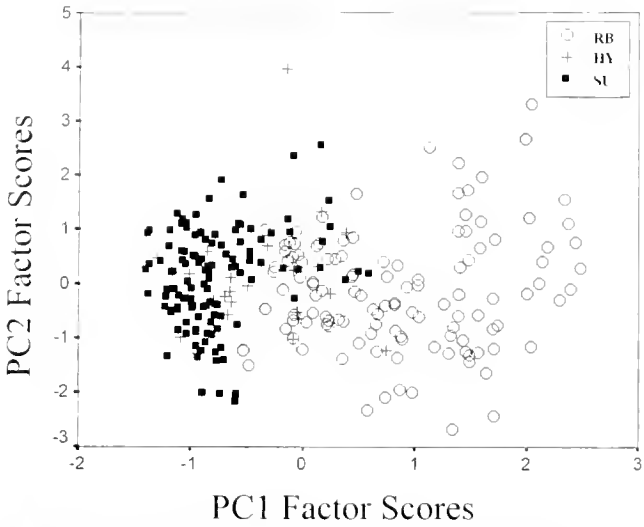

Figure 3. Relationship between principal component (PC)1 and PC2 factor scores in adult females.

ties) from wild populations of Galician *L. saxatilis* populations. The results clearly show that there is some genetic variability under the shell traits measured (Tables 1 and 2; Fig. 4). We found a mean narrow-sense heritability of 0.102 (mean of significant offspring–mother heritabilities) and a mean broad-sense heritability of 0.489 (mean of significant full-sib heritabilities) for the shell traits studied in our populations of *L. saxatilis*. However, our quantitative estimates may be somewhat biased by nongenetic effects. The resemblance between mother and offspring may underestimate the heritability due to scale effects of individual variables because adult and embryo measurements differ in one order of magnitude. Besides, if a small percentage of the embryos studied were half sibs rather than full sibs, these heritability estimates would be underestimated as well. On the other hand, the resemblance between full sibs may overestimate the heritability because it also includes the common environmental component. Maternal

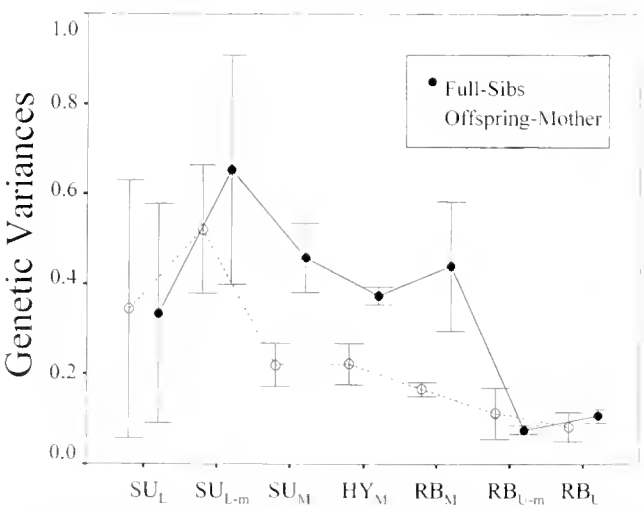

Figure 4. Trends of genetic variances (and their standard deviations) across shore levels and samples for principal component (PC)1 from full-sib correlation and offspring–mother regression analyses. SU, smooth and unbanded; HY, phenotypically intermediate; RB, ridged and banded; L, lower shore; L-m, lower mid-shore; M, mid-shore; U-m, upper mid-shore; U, upper shore.

effects could bias both estimating methods simultaneously, but this would usually occur in traits that are directly related to an embryo's fitness rather than to morphological traits. Maternal effects are assumed to be important mainly in vertebrates (Falconer & Mackay 1996). Due to the possible bias mentioned above, perhaps a more realistic narrow-sense heritability estimate would be the average between both estimates (0.296). Similar heritabilities (slightly larger) were found for morphological traits in other littorinids (Boulding & Hay 1993) and *Drosophila* (Houle 1992). The coefficient of genetic variation obtained for T and S were of similar magnitude than that obtained by Newkirk and Doyle (1975) in *L. saxatilis* populations from Halifax. These results complement our previous adaptive interpretation with respect to the Galician shell trait polymorphism by disruptive natural selection favoring each morph at different shore levels (Johannesson et al. 1993, Rolán-Alvarez et al. 1997).

The additive and genetic variability of different shell traits varied across the environmental gradient (the hybrid zone; see Table 2 and Fig. 4). In fact, upper and upper mid-shore populations showed lower additive or genetic variability than the rest of populations (Table 2; Fig. 4). These differences could be partially caused by phenotypic plasticity, a term used to include all nongenetic changes in the phenotype (genotype–environmental interactions, for example) that improve individual fitness. There are, in fact, important differences between upper and lower shore levels for many physical and biological parameters (Johannesson et al. 1993, Rolán-Alvarez et al. 1997). Phenotypic plasticity has been also shown in other littorinids (Kemp & Bertness 1984, Boulding & Hay 1993) and may have a nontrivial role in many *L. saxatilis* polymorphisms (Boulding 1990). However, the differences in genetic variances across shore levels were observed after correcting for scale effects (using both the coefficients of variation and the logarithmic transformed variables). This transformation may remove or at least diminish most interaction terms (including genotype–environment interactions) contributing to the resemblance between relatives (Falconer & Mackay 1996). Furthermore, we have obtained a significant correlation between the coefficient of genetic (estimated from full-sibs correlation) and the coefficient of additive variation (estimated from offspring–mother regression). These facts may suggest that the differences in coefficient of variation across shore levels may not be significantly biased by phenotypic plasticity, maternal effects, or other factors because the only expected component in common between these two kinds of estimates is the additive variance component. In summary, upper shore populations showed smaller genetic variability than the rest of populations. We can hypothesize that natural selection (which can erode the genetic variability of a population; see Newkirk & Doyle 1975, Falconer & Mackay 1996) may act stronger (at least in summer) on upper shores than on mid/lower shores (perhaps due to sun and desiccation stresses as well as crab predation; see Rolán-Alvarez et al. 1997). Nevertheless, to properly quantify genetic variances and phenotypic plasticity, we should do a breeding laboratory experiment to partition the familiar variance in different causal components (e.g., additive and dominance components, maternal effects, or genotype–environmental interactions).

Another interesting trend is observed on the mid-shore, where hybrids had coefficients of genetic variation or coefficients of additive variation intermediate or even lower than the same estimates in both pure morphs (Table 2; Fig. 4). These differences between morphs (RB vs. SU) were maintained when comparing populations from different habitats (upper vs. lower shore) and

populations from the same habitat (RB vs. SU from mid-shore), which suggests that the differences may not be caused by genotype–environment interactions. The observed trend was the opposite of that we would expect under hybridization of genes fixed at different shore levels or ecotypes (RB vs. SU ecotypes from mid-shore), assuming that hybrid populations are stable and at least partially fertile (Johannesson et al. 1993, Johannesson et al. 1995, Rolán-Alvarez et al. 1997, Rolán-Alvarez et al. 1999). One explanation for the low frequency of hybrids would be that hybrids are rather true F_1 hybrids unlikely to produce viable progeny. In such circumstances, the population of hybrids would be replaced every generation and would have lower genetic variability. However, embryos from female F_1 hybrids are actually F_2 hybrids; thus, we have estimated our genotypic variances at least from F_2 hybrid populations. In summary, the results suggest that several factors may be eroding the genetic variability of the hybrid populations (probably a complex mixture of hybrid genotypes). The main factor may be natural selection, with hybrids adapted to microhabitats slightly different from those of both pure ecotypes. This process of adaptation will necessarily cause some loss of the original genetic variability in the hybrid population (Falconer & Mackay 1996). A similar effect has been described in other hybrid zones; although endogenous hybrid unfitness may be the most common process on hybrid zones (tension zones sensu Barton & Hewitt 1989), hybrid amelioration (sensu Ritchie & Hewitt 1995) could explain these results if some hybrid genotypes are favored with respect to others on the mid-shore (eroding their overall genetic variability). We are presently involved in laboratory experimentation attempting to obtain F_1 hybrids and quantitative genetic estimates for the same shell traits presented above, which may help to resolve many of the questions regarding the dynamics of this hybrid zone.

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DIET IN MANGROVE SNAILS: PRELIMINARY DATA ON GUT CONTENTS AND STABLE ISOTOPE ANALYSIS

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ABSTRACT Microscopic analysis of gut contents performed on three *Littoraria* species from mangrove forests in Thailand revealed differences in diet among species. Analysis of carbon and nitrogen stable isotopes was used as an alternative way of tracing food sources. *Rhizophora* leaves, scrapings from both leaf and prop-root surfaces, and local particulate organic matter (POM) were well separated on the basis of their $\delta^{13}C$ and $\delta^{15}N$ values. In contrast, the three *Littoraria* species exhibit considerable overlap and scatter in both carbon and nitrogen isotope ratio values, suggesting that the snails are opportunistic feeders sharing similar food resources. The wide range of $\delta^{13}C$ values of *Littoraria* (−17.2‰ to −26.3‰) is consistent with carbon assimilation from multiple sources (epiphytes from leaves and prop roots, suspended POM, and *Rhizophora* detritus). *Littoraria intermedia* and *L. pallescens*, the smallest species, had similar $\delta^{13}C$ values, whereas *L. scabra* was significantly more ^{13}C depleted. A diet of microalgae and cork cells from prop roots could explain this pattern, with *L. scabra*, being larger, consuming relatively more cork cells. However, only a few of the *L. scabra* and *L. intermedia* individuals had $\delta^{15}N$ values consistent with such a diet, and the remaining *L. scabra* and *L. intermedia* and all *L. pallescens* individuals were too depleted, indicating that these individuals must derive a significant amount of their food from a strongly ^{15}N-depleted source. Such a source is present on *Rhizophora* leaf surfaces ($\delta^{15}N = 0.30 \pm 0.05$; $n = 2$). Some very low values of *Littoraria* $\delta^{15}N$, down to −7‰, indicate that some individuals have assimilated a yet unknown, highly ^{15}N-depleted food source or that other unknown fractionation processes are involved.

KEY WORDS: *Littoraria*, diet, mangroves, stable isotopes, $\delta^{13}C$, $\delta^{15}N$

INTRODUCTION

Mangrove snails of the littorinid genus *Littoraria* are found throughout the tropics with an especially large number of species in the Indo-Pacific region (Reid 1986, Reid 1989). Indo-Pacific species all have planktonic larvae and spend their adult lives on stems, prop roots, and leaves of the trees. Like most littorinids they feed on biofilms, and the mangroves presumably act merely as substrates.

Littoraria scabra (L., 1758), *L. intermedia* (Philippi, 1846), and *L. pallescens* (Philippi, 1846) have been studied in Thailand, where their stomachs have been found to contain fungal hyphae and spores, among other items (Christensen 1998). *L. scabra* and *L. intermedia* live on stems and prop roots of mangroves with *L. scabra* found lower and usually on the seaward edge of the forests. *L. pallescens* is found mostly on the leaves of mangroves where *L. intermedia* is also occasionally seen. *L. pallescens* feeds on the leaf surface without damaging the leaf epidermis, although crescent-shaped necrotic marks may be seen on leaves where the snails rest during the day. However, Ohgaki (1990) reported radular marks on leaves of *Rhizophora stylosa*. The snails move up and down with the tides and come in direct contact with the water only when releasing offspring. The zonation of the three species may be seen as degrees of terrestrialization of snails with marine origins, and it is therefore interesting to compare their diets.

Direct microscopic analysis of gut contents may reveal the range of food items ingested by animals, but the method has certain drawbacks. First of all, the method will depend strongly on the skills of the observer with respect to identifying fragmented objects. Indeed, most of the material in the gut contents of animals may not be visually identifiable. Hard structural tissues may be more easily identified than soft tissues, resulting in a bias in the relative importance of items, and this bias may be further enhanced by differences in degradability. Finally, gut contents analysis does not reveal the extent to which food items are actually assimilated.

Stable isotope analysis of animal tissues can provide alternative information on sources of food. The stable carbon isotope profile of animal tissues resembles that of the food taking into account a fractionation of about 1‰ per trophic level. Thus, for food sources differing in carbon isotope profile, the isotopic composition of the tissue may ideally indicate the relative contribution of each source to the diet. With respect to nitrogen, animals are usually enriched in ^{15}N (about 3‰) relative to the diet (Michener & Schell 1994).

The purpose of this study was to provide some preliminary information on diet characteristics of these species and to serve as a possible starting point for more detailed analyses.

MATERIALS AND METHODS

The samples analyzed were collected on different occasions; however, all samples were taken during the south-west monsoon (July–September) at the island of Phuket, Thailand. Gut contents analysis was performed on a sample (Sample 1) of snails collected on *R. apiculata* mangroves at Chalong Bay and dropped into 70% ethanol (five individuals of each species were analyzed). Another sample (Sample 2) was collected on *Avicennia marina* (*L. scabra* and *L. intermedia*) at Tang Khen Bay and *Rhizophora* (*L. pallescens*) at Chalong Bay and fixed in 70% ethanol after cracking the shell (10 individuals of each species). The contents of the stomachs were removed under a dissecting microscope, smeared onto microscope slides, and imbedded in glycerol gelatin. From each snail three smears were prepared, and from each smear three fields were scored by ocular grid (81 intersections per field). Objects at each intersection were classified (i.e., 729 scores per individual).

Snails were collected for carbon and nitrogen stable isotope analysis from *R. apiculata* mangroves at Chalong Bay and kept cool for 3 days in plastic containers during transport to Denmark

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before being frozen. For analysis the soft tissue was separated from the shell and operculum and lyophilized prior to being ground with a mortar and pestle. Prior to analysis the tissue was acidified (10% HCl) to ensure the removal of any carbonate debris, rinsed with distilled water, and then freeze-dried again. Analysis of carbon and nitrogen isotopes was also performed on stomach contents removed from snails that had been fixed in 70% ethanol (Sample 1), but only nitrogen results are presented.

Potential food sources were collected from *R. apiculata* mangroves at Chalong Bay. *Rhizophora* leaves were picked from trees, dried at 60°C, and ground for stable isotope analysis. Leaf surfaces were also carefully scraped with a surgical blade without damaging the epidermis. Scrapings from about 50 leaves were pooled in each sample and dried at 60°C before analysis. Wetted surfaces of prop roots and stems were lightly scraped with surgical blades. These scrapings were taken from random spots distributed within the range occupied by the snails, and the obtained material was shaken in a bottle with distilled water and filtered through 250-μm and 63-μm filters and finally onto pre-combusted Whatman GF/C-filters (Whatman Intl. Ltd., Maidstone, UK). The three fractions were dried at 60°C along with nonfractionated scrapings. Particulate organic matter (POM) was filtered from the waters of the bay onto precombusted Whatman GF/C filters and dried at 60°C.

Samples were prepared and analyzed as reported by Handley et al. (1991, 1993). The mass spectrometric analyses were done on a Europa 20-20 IRMS with an ANCA-SL sample converter (PDZ Europa, Cheshire, UK). A routine precision of approximately 0.1‰ for both C and N for invertebrate samples have been obtained. Stable isotope ratios are reported in standard δ notation as $\delta I = [(R_{sample}/R_{standard}) - 1] \times 1{,}000$, in units of per mil, where I is the element in question, and R is the ratio of the heavy to the light isotope. Standards were a CO_2-C standard previously calibrated against the universal Pee Dee Belemnite standard and atmospheric nitrogen.

RESULTS

The three *Littoraria* species exhibited clear differences in composition of the stomach contents with respect to identifiable components (Fig. 1). However, the major part of the stomach contents could not be identified microscopically. Cork cells from mangroves contributed significantly to the stomach contents of *L. scabra* and was also the dominant identifiable item in the stomachs of *L. intermedia*. In *L. pallescens*, fungal hyphae and spores were the most prominent identifiable objects, but these items were also present in the other two species. Diatoms, other algae, and cyanobacteria were present in all species, but only in *L. scabra* and *L. intermedia* could algae in any significant amounts be identified. Kruskal-Wallis tests revealed significant differences among species for all food items except diatoms (Sample 2): hyphae: $K = 15.8$, $P < 0.001$; spores: $K = 21.3$, $P < 0.001$; other algae: $K = 10.9$, $P = 0.004$; cork cells: $K = 19.1$, $P < 0.001$ (d.f. = 2 in all cases).

Stomach contents from snails that had the shell cracked had a higher diversity of identifiable items than snails that had been dropped directly into 70% ethanol, but also in the latter (Sample 1) were there clear differences between species, with cork cells being most prominent in *L. scabra* and fungal hyphae most prominent in *L. pallescens*.

Isotopic signatures of the soft tissue of the three *Littoraria* species exhibited considerable individual variation (Fig. 2). In *L. scabra* the mean $\delta^{13}C$ value was −24.22‰, and the range was −26.34 to −22.67‰ ($n = 16$). *L. intermedia* had a mean $\delta^{13}C$ of −22.51‰ and a range of −24.82 to −20.21‰ ($n = 16$), and *L. pallescens* had a mean $\delta^{13}C$ of −22.45‰ and a range of −24.86 to −17.27‰ ($n = 15$). A Kruskal-Wallis test revealed significant differences among species ($K = 9.21$; $P = 0.01$; d.f. = 2), with *L. scabra* being significantly more ^{13}C depleted than the other two species. Also, nitrogen isotopic signatures of the three snail species were highly variable. *L. scabra* had a mean $\delta^{15}N$ of 1.89‰ and a range of −4.64 to 6.11‰ ($n = 16$). *L. intermedia* had a mean $\delta^{15}N$ value 1.43‰ and a range of −6.97 to 6.80‰ ($n = 16$), and *L. pallescens* had a mean $\delta^{15}N$ of −1.80‰ and a range of −6.12 to 2.00‰ ($n = 15$). Differences among species were significant ($K = 9.42$; $P < 0.01$; d.f. = 2), and *L. pallescens* was significantly more ^{15}N depleted than *L. scabra* and *L. intermedia*; the latter showed the largest individual variation.

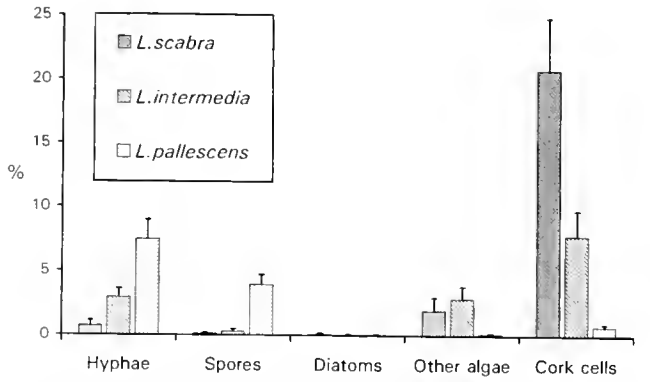

Figure 1. The occurrence of identifiable items in stomachs of three *Littoraria* species expressed as percentages (mean + standard error) of the total number of objects observed under an ocular grid in microscopic smears. Items include fungal hyphae and spores, diatoms, other algae (including cyanobacteria), and cork cells from mangroves. Animals fixed in 70% ethanol after cracking of the shell (Sample 2). *Littoraria pallescens* collected on *Rhizophora apiculata*; *L. scabra* and *L. intermedia* collected on *Avicennia marina*.

Isotope analyses performed on stomach contents of snails fixed in 70% ethanol resulted in a mean $\delta^{15}N$ of 1.33‰ (0.22 to 3.52‰; $n = 7$) in *L. scabra*, a mean $\delta^{15}N$ of 2.38‰ (−1.91 to 7.31‰; $n = 9$) in *L. intermedia*, and a mean $\delta^{15}N$ of −1.24‰ (−2.91 to −0.19‰; $n = 9$) in *L. pallescens*. Again, *L. pallescens* was the most ^{15}N depleted, and *L. intermedia* was the most variable of the species.

Rhizophora leaves, scrapings from both leaf and prop-root surfaces, and local POM were well separated on the basis of their $\delta^{13}C$ and $\delta^{15}N$ signatures (Fig. 2). POM was the least ^{13}C depleted of the sources, and *Rhizophora* leaves, leaf scrapings, and the >250-μm fraction of the prop-root scrapings were the most depleted. Leaf scrapings were highly depleted in ^{15}N compared with the other sources. A few of the *L. scabra* and *L. intermedia* individuals had $\delta^{15}N$ values consistent with a diet of mixed mangrove and POM, whereas the remaining *L. scabra* and *L. intermedia* and all the *L. pallescens* were too ^{15}N depleted.

DISCUSSION

Although only small amounts of the stomach contents could be identified, the differences among species with respect to the identifiable fraction of the diet appear to reflect true differences in diet. Whether the differences are solely a result of differences in com-

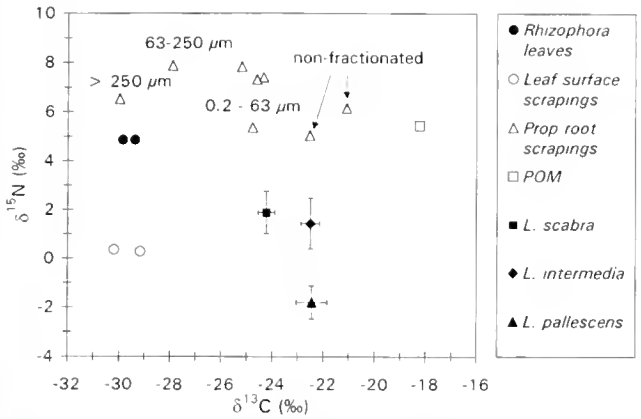

Figure 2. Stable isotopic carbon and nitrogen signatures of the soft tissue of three species of *Littoraria* collected on *Rhizophora apiculata* (means ± standard error of means) and of some potential sources of food for these snails presented as individual sample measurements.

position of the substrates upon which the snails feed (i.e., a result of zonation) or of the snails' ability to actively select among available items cannot be discerned on the basis of available data. Neither can the extent to which different items contribute to assimilated matter in the three species. Cork cells, fungal hyphae, and spores are major identifiable structural components of the diet, but whether they contribute significantly to the snails' energy budget is unknown. Fungal material in the diet was also reported by Kohlmeyer and Bebout (1986) in *L. angulifera* and by Newell and Bärlocher (1993) in *L. irrorata*.

The difference between snails that had their shells cracked prior to fixing and those fixed whole with respect to diversity of items is probably a result of continued breakdown of easily degradable food items in those snails dropped whole into ethanol. This underlines the importance of rapid and effective fixing of material for stomach analysis. The diet analyses demonstrate that there are differences between species as far as structural components in the gut contents are concerned, but these differences should not be overinterpreted because only a fraction of the gut contents can be identified.

The considerable overlap and scatter in isotope ratio values suggest that the snails are opportunistic feeders and that they to some extent share food resources. The wide range of *Littoraria* $\delta^{13}C$ values (−26.3 to 17.3‰) suggest carbon assimilation from multiple sources (epiphytes from leaves and prop roots, deposited POM, and *Rhizophora* detritus). *L. intermedia* and *L. pallescens*, the smallest species, had identical mean $\delta^{13}C$ values, whereas *L. scabra* was significantly more ^{13}C depleted. A diet of microalgae and cork cells from prop roots could explain this pattern with *L. scabra*, which is larger and consumes relatively more cork cells. However, all three species were on average more ^{15}N depleted than these food sources. *L. pallescens* had a significantly lower mean $\delta^{15}N$ value than the other two species, and it is clear that it does not derive its food directly from the mangrove leaves upon which it lives. It must derive a significant amount of its food from a strongly ^{15}N-depleted source. Such a source was present in scrapings from leaf surfaces ($\delta^{15}N = 0.3 \pm 0.05$‰; $n = 2$), but it is as yet unknown what it represents and how it is related to the diet of the snail.

Rodelli et al. (1984) reported stable carbon isotope ratios in plants and animals from Malaysian mangrove forests. They found a $\delta^{13}C$ of −27.2‰ in *R. apiculata*, which is acceptably close to our value of −29.6‰ ($n = 2$). In *L. melanostoma* they found a $\delta^{13}C$ of −24.6‰ (mean of 3) comparable to that of *L. scabra* in our study, and a value of −21.5 (mean of 10) in *L. undulata* (a rock-dwelling species). They did not report nitrogen isotope ratios.

L. irrorata in a North Carolina salt marsh had $\delta^{13}C$ values of −16.6 to −15.1‰ and $\delta^{15}N$ values of 2.2 to 3.7‰ (diet 0.1 to 3.8‰) (Currin et al. 1995), and in a Kenya mangrove forest the herbivorous snail *Terebralia palustris* had a $\delta^{13}C$ value of −24.23‰, similar to that of its presumed mangrove leaf diet ($\delta^{13}C = -24.28$‰), and a $\delta^{15}N$ signal consistent with the normal pattern of enrichment relative to the diet (Marguillier et al. 1997). If we assume that the on average very depleted ^{15}N signatures of the studied *Littoraria* tissues are not in conflict with the generally observed 3‰ enrichment per trophic level in animals (Owens 1987, Michener & Schell 1994), we must conclude that all of the three *Littoraria* species have assimilated a yet unknown and very ^{15}N-depleted food source, but other unknown fractionation processes may be involved.

The fact that the snails were kept alive for 3 days before being frozen could be invoked as a source of ^{15}N depletion. However, it is difficult to identify a mechanism leading to this result. First of all, such a mechanism should affect individuals differently because not all individuals were highly depleted. Furthermore, starvation is known to lead to ^{15}N enrichment of the tissues (e.g., Hobson et al. 1993), and, finally, stomach contents had equally ^{15}N-depleted signatures, again with *L. pallescens* being on average the most depleted. The ^{15}N signatures of stomach contents were obtained from snails that were killed without delay by dropping them into 70%ethanol, a procedure that is expected not to affect nitrogen isotope ratios. Mangrove snails may stay inactive for days during dry periods, so being deprived of food for 3 days is not entirely unnatural for them.

A purely hypothetical scenario explaining the ^{15}N depletion is that nitrogen excreted by the snails (uric acid) is recycled (e.g., through fungi), involving fractionation (depletion), and that the fungi subsequently are ingested by the snails. Supporting such a hypothesis is the fact that excretion products (ammonia, urea, and uric acid) are depleted compared with the dietary source and the tissues of the animals excreting them (Gannes et al. 1997) and that fungi are most prominent in the diet of the most ^{15}N-depleted species (*L. pallescens*). Fungi are among the microorganisms known to degrade uric acid (Kieslich 1976). Microorganisms like the cyanobacterium *Anabaena* grown on nitrate or ammonia show large fractionations and ^{15}N depletions (Macko et al. 1987).

The results underline the usefulness of multiple isotope analyses. A more limited conclusion would have been reached had the analysis been based on gut contents and stable carbon isotopes alone. The wide range of isotope ratios within species further stresses the importance of large samples in food chain studies. Considerable bias or loss of information may result if one attempts to deduce trophic relationships based on pooled samples of three or four individuals. During the last three decades, most isotopic studies have been based on small samples under the assumption that there is insignificant variation between individuals within species. Our data demonstrate that this is not always the case and that variation can be large, possibly due to small-scale heterogeneity in the occurrence and accessibility of food items. Detailed studies are needed to explain the highly ^{15}N-depleted tissues and the range of variation in N isotope ratios in these snails.

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SHELL SIZE VARIATION IN *LITTORINA LITTOREA* IN THE WESTERN SCHELDT ESTUARY

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ABSTRACT *Littorina littorea* was collected along a salinity gradient in the Scheldt estuary, located in the South of the Netherlands. Its morphological population structure was investigated to see whether salinity was correlated with shell size and shell weight. Shell size did not increase along the salinity gradient, as was expected, but rather showed a clear size transition between two salinity ranges (i.e., 10–20‰ and 21–30‰). Animals attain their largest size within a salinity range of 21–30‰. Relative shell weight did not vary consistently with salinity.

KEY WORDS: environmental stress, estuary, *Littorina littorea*, salinity, Scheldt river, shell size

INTRODUCTION

Although littorinids show high levels of intraspecific shell polymorphisms (see Reid 1996 and references therein), *Littorina littorea* (Linnaeus, 1758), the largest species in the genus, shows relatively little morphological variation (Janson 1987). However, morphological differences were noted between populations of *L. littorea* on the West Somerset coast (Crothers 1992). Along this coastline, a weak correlation was found between shell shape, as expressed by the shell length/aperture length ratio, and wave exposure (Crothers 1992). The differences were explained by differential growth and/or survival rates in response to the effects of wave exposure (Crothers 1992). In contrast, Janson (1987) found almost identical shapes between exposed rocky and boulder shore specimens. Apparently, the only consistent shell variation is found between marine and sheltered brackish forms, with the latter being smaller and thinner-walled (see Reid 1996). This variation is supposed to be ecophenotypic (Reid 1996) because *L. littorea* is a planktonic developing animal that is presumed to have a high dispersal and gene flow potential (Janson 1987, Reid 1996), minimizing the likelihood of selection as a possible impetus for the observed shell variation (Chapman 1995). Nevertheless, predation experiments with the oystercatcher *Haematopus ostralegus* have shown that the aperture size of *L. littorea* may be susceptible to selection, although field observations have never confirmed these experimental results (Robertson 1992).

L. littorea is widely distributed, occurring in the eastern (White Sea to southern Portugal) and western (Labrador to Virginia) Atlantic (Reid 1996). Unlike other littorinids, it does not solely occur on hard substrates but is also able to crawl over sand and soft mud (references in Reid 1996). This ability, along with its planktonic development and its tolerance to low salinity conditions (9.5‰), enables it to penetrate far into estuaries (Reid 1996).

In the Scheldt estuary, situated in the south of The Netherlands, *L. littorea* is found from Vlissingen (i.e., rivers' mouth) to Bath, 50 km inward from the mouth, where it occurs along a gradually decreasing salinity gradient, ranging from marine to brackish (Fig. 1). The Scheldt estuary thus forms an ideal setting to test whether salinity is indeed correlated with the shell morphology of *L. littorea*. If, under brackish conditions, *L. littorea* has a smaller, thinner-walled shell, then we expect a shell-size, weight gradient in the estuary following the salinity gradient.

MATERIALS AND METHODS

On 8 August 1998, *L. littorea* was collected at seven sites along the western Scheldt estuary, covering its entire range in the western Sheldt (Fig. 1). These sites included, in order of increasing salinity: Bath, Waarde, Hansweert, Hoedekenskerke, Ellewoutsdijk, Borssele, and Vlissingen (Fig. 1). One population was collected at each site. Each sample consisted of 40 animals. Each of the 280 specimens was morphometrically characterized. Five shell traits were measured to the nearest 0.05 mm using a caliper: shell height (HS), shell width, aperture height, aperture width, and shell-top height (De Wolf et al. 1997). In addition, total wet weight (i.e., shell + soft body parts) and body wet weight (soft body parts) were determined to the nearest mg, and all individuals were sexed on the basis of the presence or absence of a penis.

A seven-by-two contingency table was constructed to test whether the sex distribution differed from site to site, employing the Metropolis algorithm to obtain unbiased estimates of the exact *P* value (Miller 1997). Morphometric patterns were investigated by means of a two-way multivariate analysis of variance (MANOVA), contrasting the fixed factor "sex" with the random factor "sampling site." Morphological patterns were further investigated by means of a standard canonical discriminant analysis (CDA). Finally, an analysis of covariance (ANCOVA) of shell weight was performed using shell height as a covariate. Of special interest in this analysis is the interaction with the covariate because it tests whether the slopes of the shell weight on shell length are homogenous for the seven sites. Except for the contingency table analysis, all statistical analyses were performed using the software package Statistica v. 5.0 (Statsoft 1995).

RESULTS

At each site, except for Hoedekenskerke, males outnumbered females (Fig. 1). The number of males differed significantly at the different sites ($P = 0.0003$). The results of the two-way MANOVA are summarized in Table 1. A significant part of the total variation can be explained by the random factor "sampling site" (Table 1), whereas the fixed factor "sex" did not contribute significantly, nor did the interaction (Table 1)

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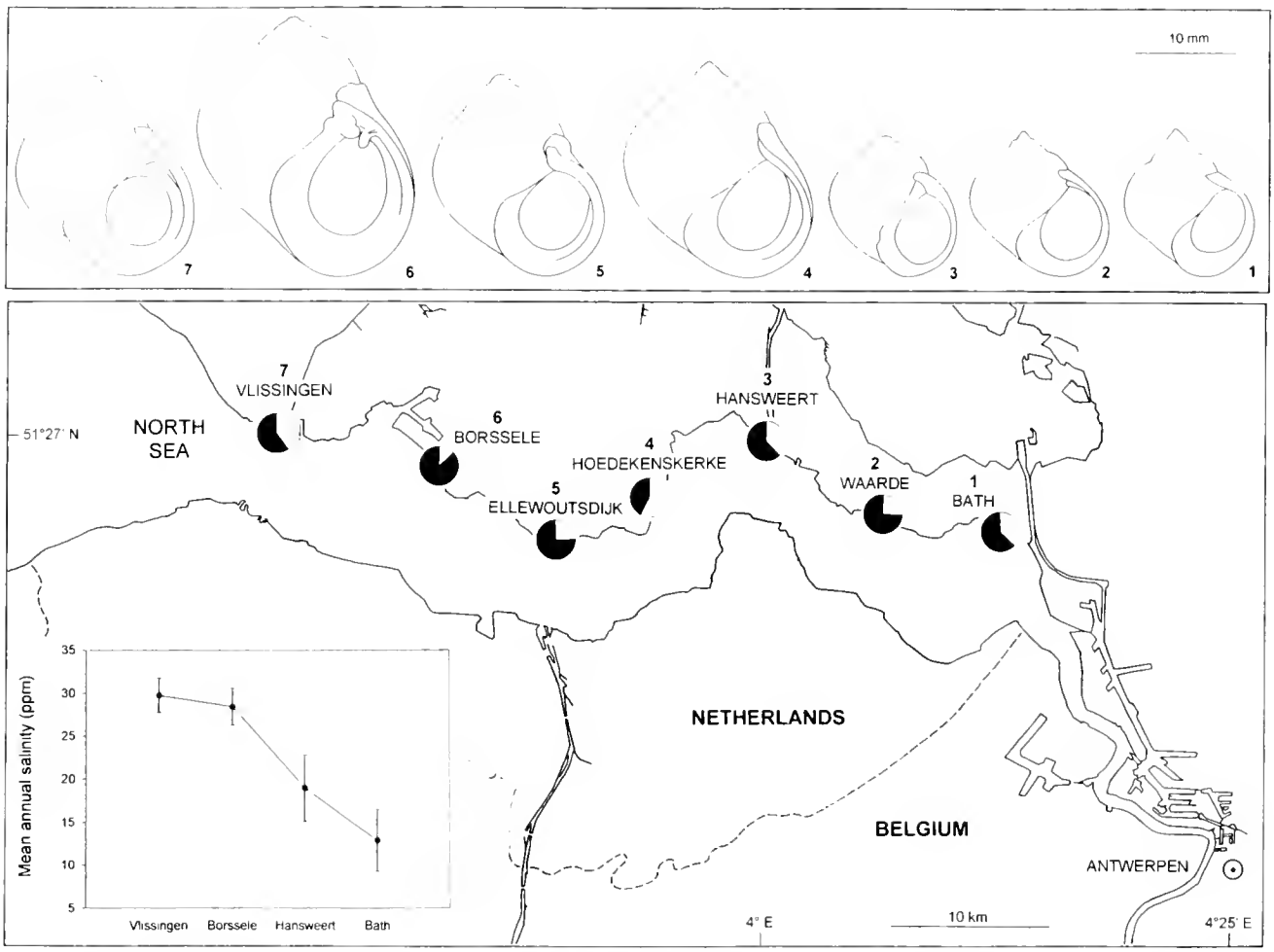

Figure 1. Sampling area, sites, and shells typical of each of the sampling sites (sites 1–7). Pie diagrams represent number of males (black zone) and females (white zone) collected at each site (n = 40). The line graph represents mean salinity values and standard deviations along the Scheldt estuary on the basis of seasonal measurements from 1990-1997.

These results are illustrated in Figure 2, where the mean shell height and standard deviations for males and females are plotted for each sampling site. Mean shell height values for both sexes overlap but simultaneously reveal a structuring at the sampling site level. Mean shell height of specimens collected at the less marine-like sites (i.e., salinity range 10–20‰: Fig. 1) are on average smaller compared with the shell height of specimens collected more downstream at a salinity range of 21–30‰ (Fig. 1). However, due to individual variation this observation is merely a trend because post-hoc Sheffé tests failed to significantly discriminate both groups.

Given that, with respect to the measured shell characteristics, males do not differ significantly from females, a single CDA was performed without considering the factor "sex." The mean values of the first two canonical variables (CV) are used to plot all sampling sites, as shown in Figure 3. The first CV describes 64.83% of the total variation and is mainly an expression of shell height (Table 2; HS = −1.11614). Shell height decreases with decreasing CV1 values, discriminating the different sampling sites, so that specimens collected at the least marine-like sampling sites are in general smaller than specimens collected at more marine-like sites (Fig. 3). The second CV describes an additional 19.83% of the total variation and is mainly an expression of the shell weight (Table 2; SW = 1.91514). Shell weight increases along the positive CV2 axis. Four groups can be distinguished along both CV axes: Bath and Waarde, consisting of specimens with small and light shells; Hansweert, consisting of specimens with small and relatively heavy shells; Ellewoutsdijk and Vlissingen, consisting of specimens with intermediate-sized shells and intermediate shell weights, and Borssele and Hoedekenskerke, consisting of specimens with larger and heavier shells. Hence, relative shell weight does not follow the salinity gradient. This is also illustrated in the ANCOVA, where the regression slopes of the shell weight on shell height are not homogeneous at the different sampling sites (Table

TABLE 1.

Results of the two-way MANOVA, contrasting the random factor "sampling site" and the fixed factor "sex."

Effect	Wilks' λ	df1	df2	P value
Site	0.183218	42	1,227	<0.0001
Sex	0.957741	7	261	0.1230
Site × Sex	0.844134	42	1,227	0.3448

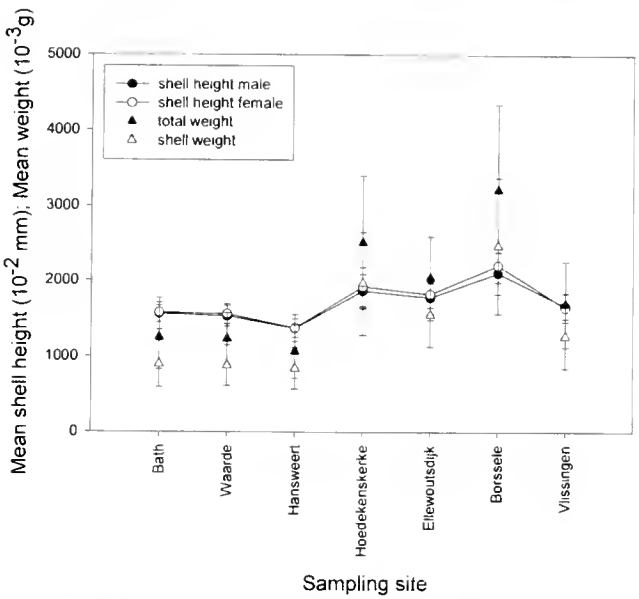

Figure 2. Mean shell height (HS), total weight (TW) (i.e., soft body weight + shell weight), shell weight (SW), and standard deviations for males and females of *Littorina littorea* collected at the seven sites.

3) and in Figure 2, where mean shell weight, mean total weight, and mean shell height are plotted at the different sampling sites.

DISCUSSION

As was predicted, *L. littorea* had smaller and lighter shells in more brackish conditions. However, shell size did not decrease clinally away from the sea, following the salinity gradient. Instead, specimens were either small (approximately <15 mm) or large (approximately >19 mm). No intermediate-sized animals were found at sites having intermediate salinity levels (i.e., ±15‰). If salinity is indeed an environmental factor that affects shell size in *L. littorea*, it seems that its effect is either present or absent with a threshold salinity value of approximately 15–20‰. Above this salinity threshold, animals are able to attain a large shell; below this value, animals never have comparable shell sizes. In either case it must be clear that, at brackish sites, *L. littorea* is likely to encounter less favorable living conditions, which might result in a decrease of growth and/or survival rate (Crothers 1992), shifting the less marine-like populations toward smaller-sized individuals. It must also be noted, however, that the largest shells were not recorded at Vlissingen (i.e., highest salinity) but at Borssele (i.e., second highest salinity). At Borssele, specimens were collected in the direct vicinity of a nuclear power plant. Possible water temperature differences, due to the outflowing cooling water, might affect the species shell growth. Indeed, larger shells can be produced at higher temperatures due to the lower energy cost of calcification because calcium carbonate dissolves less well at higher temperatures (Graus 1974, Clarke 1983). However, on a macrogeographical scale, *L. littorea* attains its largest size in the northern parts of its distribution range (i.e., cooler waters) (Reid 1996). Clearly, further experimental work is needed to clarify and explain the possible effects of water temperature and salinity on shell deposition in *L. littorea*.

In the case of shell weight, the presumed relationship with salinity was even less clear. Moreover, it seemed unlikely that relative shell weight differences can be explained by salinity differences. The top right positioning of the Hansweert population in the CDA graph (Fig. 3) indicates that specimens with the highest relative shell weight occur under the third lowest salinity conditions.

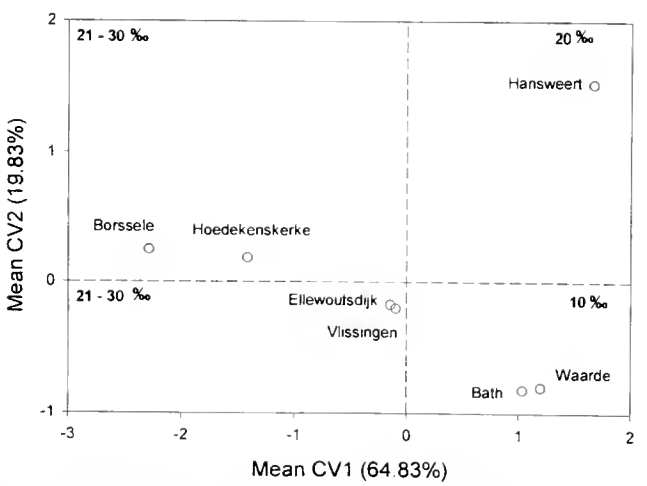

Figure 3. Mean values for the first two cannonical variables.

TABLE 2.

Standardized coefficients for the first two cannonical variables (CV) in a canonical discriminant analysis contrasting all eight sampling sites.

Dependent Variable	CV1	CV2
Shell height	−1.11614	−1.92037
Shell width	0.28414	0.81833
Aperture height	−0.71504	0.03916
Aperture width	0.68485	−1.47668
Shelltop height	0.57448	0.61353
Shell weight	−0.58913	1.91514
Eigenvalue	1.83935	0.56258
Explained variation, %	64.83	19.83

TABLE 3.

Results for the interaction of the dependent variable shell weight (SW) and the covariate shell height (HS) in the ANCOVA (i.e., test for parallelism) and corresponding regression equations at each of the seven sampling sites.

	SS	df	MS	*F* Value	*P* Value
Effect	3,811,414	6	635,235.6	20.06198	<0.0001
Error	8,454,196	264	31663.7		

Site	Regression equation	r^2
Bath	HS = 1,059.5 + 0.5614 SW	0.8928
Waarde	HS = 1,133.3 + 0.4672 SW	0.8832
Hansweert	HS = 913.2 + 0.5549 SW	0.8485
Hoedekenskerke	HS = 1,258.3 + 0.3239 SW	0.8452
Ellewoutsdijk	HS = 1,082.9 + 0.4553 SW	0.9103
Borssele	HS = 1,398 + 0.2897 SW	0.9078
Vlissingen	HS = 1,169.3 + 0.3844 SW	0.8737

Abbreviations: SS = Sum of squares; MS = Mean square.

Sexual dimorphism is common in the genus *Littorina*, with females being larger than males (Reid 1996), which is presumed to be related to growth and/or longevity differences (Reid 1996). However, sex-related shell height differences have not been found in *L. littorea* (Reid 1996), even though sexual selection for female shell size has been documented, with males preferring to mate with larger, and thus more fecund, females (Erlandson & Johannesson 1994). The fact that we did not find sex-related shell size differences is thus in agreement with what was previously found. However, our sex assignment, made on the basis of the presence or absence of a penis, leads to a sex ratio of almost 2:1 (male/female), which differs markedly from a previously published sex ratio of 1:1 (Daguzan 1977). The fact that we found twice as many males might be related to the presence of imposex—the development of male sex characteristics on females (e.g., a penis and/or vas deferens) (Bauer et al. 1997)—and/or intersex—the disturbance of the phenotypic sex determination between the gonad and genital tract—which is known to occur in *L. littorea* (Bauer et al. 1997). Imposex has never been recorded in *L. littorea*. In contrast, intersex gradually transforms the female pallial tract such that the female pallial organs are supplanted by a male prostate gland, and a seminal groove and a small penis occur (Bauer et al. 1997). The fact that penis shedding and/or regression also occur in *L. littorea* (Deutsh & Fioroni 1992) makes it even more difficult to distinguish between an intersex female and a male with a shed or regressed penis. Therefore, it could be that some specimens that were classified as males were in fact intersex females. However, female intersex expression occurs only in juvenile stages or during sexual immaturity (Bauer et al. 1997). As a consequence, intersex females are expected to have a smaller shell. Hence, if intersex females were included in the male population, they would not have increased the mean male shell height, masking possible shell size differences with the presumed larger females, but rather would have decreased the mean shell height of the male population. In any event, the occurrence of penis shedding, penis regression, and intersex make basing sex assignment in *L. littorea* on the presence of a penis unreliable. Similarly, the presence or absence of a prostate gland is also an unreliable sex-determining character (Bauer et al. 1997).

Finally, salinity is not the only environmental factor that may be correlated with the morphology of *L. littorea*. In an estuary, which is structured by a complex of gradients, a wide variety of natural and human-induced stresses are present that may affect the shell morphology of estuarine gastropods. In this respect, in the period 1981 to 1983, the Scheldt estuary was ranked among the most heavily polluted estuaries around the world for both the dissolved as well as the particulate metal phase (Bayens 1998). Dissolved metal concentrations measured at Hansweert and Vlissingen differ significantly, with Vlissingen being less polluted (Rijksinstituut voor Kust en Zee, RIKZ, pers. comm.). Concentrations of volatile organic compounds are high and decrease along the estuary as well (De Wulf et al. 1998). Clearly, pollution is an important potential stressor in the Scheldt estuary, and its effect on the morphological population structure of the estuarine organisms must be investigated.

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CARBONATE PROCESSING BY INTERTIDAL GASTROPODA ON JAMAICAN LIMESTONE SHORES

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ABSTRACT At least eight species of Littorinidae occur sympatrically on a limestone platform on the northern coast of Jamaica with little evidence of competitive displacement. Most of these, plus a cobble shore gastropod (*Planaxis nucleus*), were studied with respect to the amount of carbonate each removes from the shore while feeding. The feces of *P. nucleus* contain 92.8% carbonate. On the platform, *Nodilittorina riisei* fecal pellets contained 88.7% carbonate, *N. ziczac* 74.9%, *N. angustior* 67.0%, *N. dilatata* 88.3%, *Tectarius antonii* 91.3%, *Cenchritis muricatus* collected from the rocky substratum 74.8%, and *C. muricatus* collected from the maritime shrub *Rhachicallis americana* 18.6%. Although the rock-dwelling Littorinidae ingest different quantities of carbonate, there is no clear relationship between the amount of carbonate ingested and the position each species occupies on shore. Fecal pellet counts were made for all species. The mean numbers of pellets recovered from the rectums were: *P. nucleus* (48.7), *N. riisei* (8.56), *N. ziczac* (26.1), *N. angustior* (14.1), *N. dilatata* (22.2), *T. antonii* (22.8), and *C. muricatus* (35.3). The mean size and weight of pellets for each species were: *P. nucleus* (1.73 mm^2, 0.040 mg), *N. riisei* (1.21 mm^2, 0.028 mg), *N. ziczac* (1.43 mm^2, 0.066 mg), *N. angustior* (0.959 mm^2, 0.024 mg), *N. dilatata* (1.76 mm^2, 0.052 mg), *T. antonii* (3.23 mm^2, 0.118 mg), and *C. muricatus* (4.45 mm^2, 0.112 mg). The bioerosive impact of each species was assessed by calculating the amount of carbonate removed from the shore per individual and per species based on density and an estimated 48-h defecation cycle. Impacts were expressed in terms of both a single defecation cycle and annually. Collectively, the Littorinidae are estimated to remove at least 2,850 kg of carbonate from the approximately 500 × 24-m limestone platform annually.

KEY WORDS: *Littorinidae*, fecal pellets, carbonate, rocky shore, Jamaica

INTRODUCTION

At least eight species of Littorinidae (Lang et al. 1998, Minton & Gochfeld 2001) occupy a limestone platform on the northern coast of Jamaica, from near sea level to as much as 4 m above it. Most of them (except the tidepool-dwelling *Nodilittorina mespelum*) occupy very similar niches in broadly overlapping ranges from the splash zone to as much as 30 m inland from the sea, with little evidence of competitive displacement. Several previous studies have attempted to elucidate the roles of biotic and abiotic factors in controlling the distribution of sympatric littorinid species on hard-shore habitats, mostly with inconclusive results. Britton (1992) showed that the resistance to evaporative water loss and tolerance to thermal stress had little correlation to the position on the shore occupied by each species. He suggested, however, that dessication tolerance may be a more important limiting factor than thermal stress in dictating species position on shore. Lang (1995) found that shell sculpture and spire height in three littorinid species were unrelated to shore position and shell nodulosity. The presence of nodules on shells is widely assumed to confer a temperature regulatory advantage (Vermeij 1973); however, they could not be shown to do so. In a comprehensive survey of several ecological parameters associated with Jamaican rocky-shore littorinids, including position and direction of repose, substratum and body temperatures, nature of attachment to the substratum, microhabitat (within crevices or not), and availability of moisture in association with the substratum, Lang et al. (1998) found few significant correlations between these parameters and the positions species occupy on the shore. Furthermore, there were few species-specific differences for most of the parameters examined. Substratum moisture, however, was modestly predictive of which species might be expected on a portion of the shore, with *N. riisei* always occupying wetted surfaces at the seaward face of the platform, *Cenchritis muricatus* always on the highest and driest areas at the back of the platform, and the other species at various, broadly overlapping positions in between.

Location on a shore is an important factor in determining the degree of stress a species will encounter, ranging from thermal and dessication exposure to what foods are available to the species (McMahon 1990, Norton et al. 1990, McMahon & Britton 1991). On the Jamaican platform shore, *N. riisei* (Mörch, 1876), *N. ziczac* (Gmelin, 1791), and *N. angustior* (Mörch, 1876) occupy the upper eulittoral zone, although the latter may also range into the lower supralittoral fringe. *N. dilatata* (d'Orbigny, 1846) and *Tectarius antonii* (Philippi, 1846) have a broad distribution across the mid- to lower supralittoral fringe, whereas *C. muricatus* (Linnaeus, 1758) preferentially occupies the upper supralittoral to the margin of maritime vegetation (Lewis 1960, Bandel 1974a, Lang et al. 1998). *Planaxis nucleus* (Bruguière, 1789) (Prosobranchia: Planaxidae), the only nonlittorinid examined in this study, does not occur on the platform but is found instead on large immobile limestone boulders awash in surf on a nearby cobble shore, identified locally as Jingle Beach.

The Littorinidae are predominantly herbivorous gastropods that rasp the substratum for food by means of a radula (Reid 1989, Norton et al. 1990). On the Jamaican rocky shore, there are differences in the quantity and quality of food sources from the splash zone to the highest parts of the limestone platform. It seems plausible, therefore, that the herbivores occupying different vertical zones have different food preferences. Low-zoned species such as *N. riisei*, in the presence of abundant moisture and epilithic algae, might rely less upon potentially harder-to-reach endolithic cyanobacteria. In contrast, the high-zoned *T. antonii*, living exclusively on rock, has little opportunity to feed upon epilithic or epiphytic algae. The higher-zoned *C. muricatus* may graze upon the stems and leaves of maritime vegetation, but when on rock, it, like *T. antonii*, must focus instead on a diet of endolithic algae and/or lichens. Peckol and Guarnagia (1989) showed that the gut of *T.*

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antonii contains considerable amounts of endolithic cyanobacteria, which occur abundantly throughout the vertical range of this species. We will show that *C. muricatus* has an alternate food source among the maritime angiosperm vegetation.

In this study, we hypothesize that diet may reflect one aspect of resource partitioning among intertidal gastropods. Rock-dwelling species that must rely heavily upon endolithic algae for food (e.g., *C. muricatus* and *T. antonii*) should also have a large quantity of non-nutritious calcium carbonate in the feces. Conversely, species living among epilithic algae (e.g., *N. riisei* and *N. ziczac*) may focus on this more easily obtained food when it is available. If so, the fecal carbonate fraction should be much less than that associated with the high-zoned species.

Members of the Littorinidae and Planaxidae are ideal models for an analysis of fecal content because they form the feces into discrete, compact, and relatively glutinous pellets that are retained in a discrete rectum until eliminated from the body. Fecal pellets of the Jamaican rocky shore Littorinidae have a similar morphology, mostly ranging from ovoid to drop-shape. They contrast sharply with the narrow, spindle-shaped pellets of the Planaxidae (Bandel 1974b). The pellets of those species living on rocky surfaces consist mainly of fine detritus, including mineral flakes and rock fragments that they scrape from the surface while rasping off algae (Bandel 1974b).

We hypothesize, therefore, that the fecal pellets of different species of herbivorous intertidal Gastropoda contain carbonate in different proportions according to their food choices and/or method of grazing. If such differences can be detected, this might suggest one method by which these species partition resources on this shore. We also assess the bioerosive impact of these species. The number and weight of the fecal pellets per individual as well as measurements of the size of fecal pellets for 50 individuals of each species were used to estimate the amount of carbonate removed from shore by these gastropods.

MATERIALS AND METHODS

Six species of Littorinidae (Gastropoda: Prosobranchia: Littorinidae) were collected from a micro-karsted limestone shore platform near the Hofstra Marine Lab at Priory, St. Ann's Parish, on the northern coast of Jamaica. Lang et al. (1998) described this shore and the distribution of the littorinids on it. *N. dilatata*, *T. antonii*, and *C. muricatus* are mid- to high-zoned nodulose species, whereas *N. ziczac*, *N. angustior*, and *N. riisei* produce relatively smooth, zebra-striped shells and occupy mid- to lower levels of the platform. A seventh species examined for this study, *P. nucleus* (Gastropoda: Prosobranchia: Planaxidae), was collected from large immobile boulders at Jingle Beach, a cobble shore located about 500 m west of the limestone platform.

All species were collected from a limestone substratum, either in repose or crawling upon it. *C. muricatus*, however, also occurred on a small maritime shrub, *Rhachicallis americana*. Individuals from this substratum were also collected and treated separately during the laboratory phase of this study. Collected individuals of all species were preserved in 70% ethyl alcohol within seconds of collection to minimize the loss of fecal pellets by natural defecation.

Fecal pellets were removed from 740 individuals (i.e., 80–130 individuals per species) (Table 1). Prior to dissection, shell measurements (shell height, shell width, aperture height, and aperture width) of each individual were determined by means of digital calipers. Each shell was then broken, the soft body was removed, and the rectum and its contained fecal pellets were excised from each body. Fecal pellets removed from the rectum were placed on a pre-weighed drying boat, their wet weight was determined, and the number extracted from each individual was counted.

The height and width of 250 fecal pellets (i.e., five from each of the first 50 individuals dissected) were determined for each species by means of a microscopic monocular micrometer. Because there was no reliable, accurate way to measure depth, pellet size was expressed as area, not volume. Means and standard deviations of fecal pellet area were calculated for all species, and a Kruskal-Wallis single factor analysis of variance (ANOVA) by ranks was performed to determine species-specific differences, if any. Linear regressions were performed to assess the within-species relationship between fecal pellet number and body size, as indicated by shell height. If these analyses indicated a significant relationship, the slopes of the regression lines could be compared by an analysis of covariance (Zar 1984) to determine if there were significant differences between species.

Fecal pellets were placed in a 55°C drying oven until a constant dry weight was attained. The means and standard deviations for fecal pellet dry weight were calculated on a per-pellet basis for all species, and these values were compared between species by means of a Kruskal-Wallis single factor ANOVA by ranks. The pellets from each individual were then assessed for meeting a critical dry weight (i.e., a minimum quantity of fecal material

TABLE 1.

Summary statistics for number of individuals dissected, total number of fecal pellet samples, number of samples containing only one individual, number of samples containing more than one individual (i.e., a pooled sample), and the mean, standard deviation, and range for the number of individuals contained in the pooled samples.

	Number of individuals dissected	Total number of samples	Number of samples with only one individual	Number of pooled samples	Number of individuals in a pooled sample		
					Mean	Standard deviation	Range
P. nucleus	113	37	0	37	3.05	0.88	2–5
N. riisei	80	3	0	3	26.7	12.2	16–40
N. ziczac	122	38	1	37	3.27	2.77	2–13
N. angustior	80	6	0	6	13.3	2.16	11–17
N. dilatata	81	20	0	20	4.15	1.18	2–6
T. antonii	130	56	4	52	2.42	0.67	2–5
C. muricatus on plants	54	45	42	3	2.67	0.58	2–3
C. muricatus on rocks	62	35	12	23	2.17	0.49	2–4

necessary to assure a sufficient, measurable sample size after acid treatment). The critical dry weight was determined to be about 4.5 mg. If the fecal pellets from an individual weighed less, they were combined with others from individuals of the same species (i.e., pooled) until the critical weight was reached or exceeded. It was necessary to pool fecal pellets for all species (Table 1), but many individuals of *C. muricatus* and *T. antonii* had sufficiently large and/or numerous fecal pellets that attained or exceeded critical weight.

Each dried fecal sample attaining critical weight (i.e., either the rectal contents of one individual or a pooled sample of pellets from several individuals within a species) was washed with de-ionized water onto a 25-mm nitrocellulose filter attached to a Millipore microfiltration apparatus. The water was drained from the apparatus with a vacuum pump, leaving the fecal pellets on the filter. Then, 1 M nitric acid was added to the apparatus and allowed to react with the calcium carbonate in the fecal sample until all effervescence ceased, indicating conversion of calcium carbonate to carbon dioxide according to the following reaction:

$$CaCO_{3\,(S)} + 2\,HNO_{3\,(Aq)} \rightarrow Ca(NO_3)_{2\,(Aq)} + CO_{2\,(G)} + H_2O_{\,(Aq)}$$

After effervescence ceased, another dose of nitric acid was added to ensure that the reaction was complete. The filter paper containing the fecal sample was thoroughly washed with de-ionized water, removed to a pre-weighed drying boat, and dried in an oven to a constant weight. The difference between the dry weights prior to and after the acid treatment is considered to be the amount of calcium carbonate removed by the acid treatment plus an assumed negligible amount of other acid-volatile or acid-soluble organic materials. This difference will be referred to subsequently as the fecal carbonate fraction or, simply, ingested carbonate.

The fecal carbonate fraction was compared between each species and between the two subsets of *C. muricatus* (i.e., those collected from limestone and those collected from plants). Means and standard deviations, expressed as proportions, were calculated for all species and were compared by a Kruskal-Wallis single factor ANOVA by ranks. Two species, *N. riisei* and *N. angustior*, required extensive pooling to produce a sample that reached the critical mass needed for analysis (Table 1). Thus, they were represented by a small number of pooled samples ($n = 3$ and $n = 6$, respectively). Accordingly, all species except these two were again compared by a Kruskal-Wallis single-factor ANOVA by ranks to assess the impact caused by extensive pooling and to eliminate any bias caused by it.

C. muricatus and *T. antonii* included both unpooled and pooled samples. Such data were compared within each species (and within each species according to habitat for *C. muricatus*) by means of a *t* test to assess the impact of pooling. The parametric *t* test was selected for comparison of within-species proportions because both normality and equality of variance tests were within required limits. All statistical tests were performed using the Sigma Stat statistical package.

On the basis of fecal carbonate estimates determined in this study, we also estimated the annual bioerosive impact of these species on the shore (1) per individual on the basis of an estimated 48-h defecation cycle (the duration from ingestion to defecation); (2) per individual per year, also on the basis of an estimated 48-h defecation cycle; and (3) total carbonate removed from this shore per species per year on the basis of density estimates by Metz (1997). Because the densities of each species varied with respect to both species and location on the shore, bioerosive impacts were calculated on the basis of mean densities within the occupied range of each species, not for the entire platform. The 48-h duration of the littorinid defecation cycle was never tested empirically. Laboratory behavior of these animals suggested, however, that the defecation cycle, although influenced by numerous extrinsic factors including duration of repose, is probably no longer than a mean 48 h and is likely much shorter. Thus, the carbonate removal calculated for each species using the 48-h estimate is probably a conservative assessment of the bioerosive impact of the littorinids. No attempt was made to estimate carbonate removal by *P. nucleus* and *C. muricatus* from plants because there were no estimates available concerning their density on shore.

RESULTS

Fecal pellets were removed from between 80 and 130 individuals of each species (Table 1). Between 20 and 56 samples, consisting of fecal pellets from one to several individuals, were processed for all species except *N. riisei* and *N. angustior*. Because the latter produced such small pellets, it was necessary to combine those from several individuals to obtain a sample sufficient for analysis. Thus, 80 *N. riisei* yielded only three pooled samples for analysis, and 80 *N. angustior* yielded only six.

Mean fecal pellet size, expressed as area (Table 2), and mean fecal pellet weight (Table 3) were calculated for each species. *N. angustior* produced the smallest fecal pellet in terms of both mean size and mean weight per pellet. *C. muricatus* produced the largest pellets with respect to mean size, but *T. antonii* had the largest pellets with respect to mean weight. The pellets produced by both of these species were significantly larger than those produced by the other species with respect to size (Table 2) and weight (Table 3). *C. muricatus* pellets also differed significantly in size from those of *T. antonii*, but not significantly, with respect to weight. Intraspecific variation in pellet size was relatively great for all species, as reflected by high standard deviations (Table 2). Dunn's pairwise comparisons following a Kruskal-Wallis single-factor ANOVA by ranks also indicated fecal pellet size to be significantly different between most species (Table 2). Variation in pellet weight within each species was slight to moderate, as indicated by standard deviation values (Table 3), and there were no significant

TABLE 2.

Comparison of fecal pellet size, expressed as area (mm^2), among all species by a Kruskal-Wallis single-factor ANOVA by ranks (α = 0.05, H = 1117.1, df = 6, P = <0.001, * = significant difference). CM, *Cenchritis muricatus;* NA, *Nodilittorina angustior;* ND, *N. dilatata;* NR, *N. riisei;* NZ, *N. ziczac;* PN, *Planaxis nucleus;* TA, *Tectarius antonii.*

	Mean fecal pellet area (mm^2)	Standard deviation	CM	NA	ND	NR	NZ	PN
C. muricatus	4.45	1.61	—					
N. angustior	0.959	0.460	*	—				
N. dilatata	1.76	0.672	*	*	—			
N. riisei	1.21	0.554	*	*	*	—		
N. ziczac	1.43	0.837	*	*	*		—	
P. nucleus	1.73	0.586	*	*		*	*	—
T. antonii	3.23	0.837	*	*	*	*	*	*

TABLE 3.

Comparison of fecal pellet weight (mg) among all species by a Kruskal-Wallis single factor ANOVA by ranks (α = 0.05, H = 161.7, df = 6, P = <0.001, * = significant difference).

	Weight per pellet (mg)	Standard deviation	CM	NA	ND	NR	NZ	PN
C. muricatus	0.112	0.044	—					
N. angustior	0.024	0.003	*	—				
N. dilatata	0.052	0.011	*		—			
N. risei	0.028	0.005	*			—		
N. ziczac	0.066	0.022	*				—	
P. nucleus	0.040	0.009	*					—
T. antonii	0.118	0.019		*	*	*	*	*

Abbreviations as in Table 2.

differences in pellet weight between *N. angustior*, *N. dilatata*, *N. riisei*, *N. ziczac*, and *P. nucleus*.

Among the species of Littorinidae, the mean number of fecal pellets per individual generally increased as mean shell size increased (Table 4; Fig. 1). The smallest littorinid (*N. riisei*) produced the fewest number of pellets, and the largest species (*C. muricatus*) produced the most pellets among the littorinids. *P. nucleus* produced significantly more fecal pellets than any of the littorinids, but each pellet was significantly smaller than that produced by any littorinid of comparable size. Linear regressions were performed to compare the number of pellets as a function of size (shell height) for each species (Table 5). Despite the general trend of increasing numbers of pellets with increasing body size for all species except *N. dilatata*, the predictive value for all regressions was poor, as reflected by very low adjusted r^2 values, ranging from a low −0.002 for *C. muricatus* to a high of 0.37 for *N. ziczac*. Accordingly, the slopes of the regression lines were not compared between species.

Samples of *T. antonii*, *C. muricatus* collected from plants, and *C. muricatus* collected from rock included both single-individual and pooled (several individuals) pellet samples. We performed *t* tests performed between the individual and pooled subsets of each group, which detected no statistically significant differences between any of them.

The proportion of carbonate (strictly speaking, acid-volatile or acid-soluble material, represented mostly by calcium carbonate) in the fecal pellets varied greatly (Table 6). *C. muricatus* fecal pellets collected from plants contained the smallest carbonate fraction (18.6%), whereas *T. antonii* and *P. nucleus* contained the largest (93.1 and 92.8%, respectively). There were also three other distinct groupings: (1) *N. riisei* and *N. dilatata* (88.7 and 88.3%, respectively), (2) *N. ziczac* and *C. muricatus* from rocks (74.9 and 74.8%, respectively), and (3) *N. angustior* (67.0%). Dunn's pairwise comparisons following a Kruskal-Wallis single-factor ANOVA by ranks also indicated that the fecal carbonate content was not significantly different between those species with the highest amount (*T. antonii*, *P. nucleus*, *N. riisei*, and *N. dilatata*) and not significantly different between those with low to middle amounts (*N. ziczac*, *C. muricatus* from rocks, *N. riisei*, *N. dilatata*, and *N. angustior*) (Table 6). The carbonate content of fecal pellets from *C. muricatus* found on plants (18.6%) differed significantly from that of conspecific individuals taken from rock as well as from that of all other species except *N. angustior* (67.0%).

When *N. riisei* and *N. angustior* were removed from the data set and the Kruskal-Wallis single factor ANOVA by ranks was performed again without them (Table 6), two new significant differences appeared between *N. dilatata* and *C. muricatus* from rocks and between *N. dilatata* and *N. ziczac*.

Two large, upper-shore species, *C. muricatus* (from rock) and *T. antonii*, processed the greatest mean amounts of carbonate per defecation cycle (3.07 and 2.44 mg, respectively) and per year (0.546 and 0.435 g, respectively) (Table 7; Fig. 2). The two smallest species, *N. riisei* and *N. angustior*, processed the smallest mean amount of carbonate per defecation cycle (0.21 and 0.23 mg, respectively) and per year (0.037 and 0.040 g, respectively). Expressed on an individual basis, *N. dilatata* also seems to have only a modest impact, processing only 1.12 mg per defecation cycle or 0.199 g per year.

These relationships are much different when population densities are considered. Population densities of the Littorinidae on this shore ranged from 80 individuals/m^2 for *N. ziczac* to 736 individuals/m^2 for *N. dilatata* (Table 7). *C. muricatus*, *T. antonii*, *N. riisei*, and *N. angustior* had densities of 128, 208, 272, and 480 individuals per m^2, respectively, within occupied ranges. On the basis of these densities, *N. dilatata* removes the largest amount of carbonate from the platform annually, 146.5 g/m^2 (Table 7; Fig. 3). *T. antonii* and *C. muricatus* from rocks had the second and third highest values, removing 90.5 g/m^2/y and 69.9 g/m^2/y, respectively. All other species had much lower values for annual carbonate removed: *N. angustior* with 19.2 g/m^2/y, *N. ziczac* with 16.9 g/m^2/y, *C. muricatus* on plants with 15.6 g/m^2/y, and *N. riisei* with 10.1 g/m^2/y.

DISCUSSION

Although the Littorinidae and Planaxidae are primarily herbivores, the focus of their feeding varies widely, often according to habitat. Species that occupy the algal gardens of the lower eulittoral may ingest the algae upon which they live. For example, *Littorina littorea* is attracted to and feeds upon *Ulva lactuca*, and *L. obtusata* is attracted to this species plus *Fucus serratus* and *Ascophyllum* spp. (Watson & Norton 1985, Watson & Norton 1987). Other littorinids browse on the surfaces of seaweeds or seagrasses, consuming epiphytic algae, fungi, or microorganisms such as diatoms, protozoa, cyanobacteria, and bacteria (Norton et al. 1990). Several littorinids, especially members of the genus *Littoraria*, browse on the surfaces of mangroves (Reid 1986, Christensen 1998). Others, such as *Bembicium auratum*, feed on

TABLE 4.

Comparison of number of fecal pellets per individual among all species by a Kruskal-Wallis single-factor ANOVA by ranks (α = 0.05, H = 341.8, df = 6, P = <0.001, * = significant difference).

	Mean number of pellets	Standard deviation	CM	NA	ND	NR	NZ	PN
C. muricatus	35.3	17.1	—					
N. angustior	14.1	7.88	*	—				
N. dilatata	22.2	10.9	*	*	—			
N. riisei	8.56	5.37	*		*	—		
N. ziczac	26.1	16.8	*	*		*	—	
P. nucleus	48.7	16.1	*	*	*	*	*	—
T. antonii	22.8	7.81	*	*		*		*

Abbreviations as in Table 2.

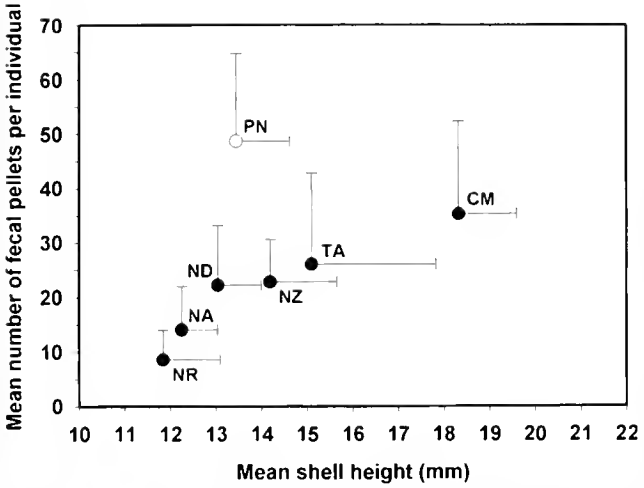

Figure 1. Mean number of fecal pellets per individual as a function of shell height (mm). Error bars are given only in the positive directions for clarity. Open circle, Planaxidae; filled circles, Littorinidae. CM, *Cenchritis muricatus*; NA, *Nodilittorina angustior*; ND, *N. dilatata*; NR, *N. riisei*; NZ, *N. ziczac*; PN, *Planaxis nucleus*; TA, *Tectarius antonii*.

the microflora in the mud beneath the mangroves (Branch & Branch 1981a). Perhaps the majority of littorinids that occupy rocky shores browse directly on rock surfaces and ingest detritus, small live animals, egg capsules, sand grains and fragments of rock, lichens, and epilithic and endolithic algae (Norton et al. 1990). Species occupying the eulittoral on rocky shores probably focus on epilithic and/or epiphytic algae with those inhabiting similar habitats sometimes seeking different foods. For example, the stomach contents of *L. planaxis*, a eulittoral snail of granitic shores in California, include microphytes such as green algae, blue-green algae, and abundant quantities of diatoms, the latter probably the primary food source (Foster 1964). On the same shore, *L. scutulata* often occurs on and grazes macrophytic algae such as *Cladophora*, *Pelvetia*, and *Rhodoglossum* (Dahl 1964).

Higher upon the shore where drier conditions are more common, epilithic and epiphytic algae usually disappear, often to be replaced by a surface film of encrusting black lichens, which are grazed upon by several species of littorinids including *N. unifasciata* in Australia (Branch & Branch 1981b). On the highest zones of tropical limestone shores, however, endolithic algae, especially cyanobacteria, are present (Peckol & Guarnagia 1989) and provide high-zoned littorinids a food source in an otherwise nearly barren habitat (North 1954, Hodgkin et al. 1959, Underwood 1984a, Underwood 1984b, Underwood 1984c). A few rocky-shore littorinids, such as *C. muricatus*, range to the fringe of maritime vegetation and derive additional nutrition by grazing upon their stems and leaves.

Planaxidae occupy bedrock or cobble shores and, like the Littorinidae, employ a radula to scrape the hard surfaces upon which they live. *P. nucleus* lives in a high-energy, mid-intertidal habitat among boulders or large rocks usually surrounded by loose, mobile stones. Planaxids generally have a large, powerful foot that grips the substrate tenaciously, preventing dislodgement by the waves or rolling stones (Houbrick 1987).

Most previous studies of intertidal gastropod fecal content have emphasized the nutritious organic fraction, not the inorganic component. Accordingly, most of these studies employed either ashing or isotope analysis to estimate the organic fraction (Black et al. 1988, Norton et al. 1990, Newell & Bärlocher 1993, Christensen 1998). For example, Black et al. (1988) examined the fecal content of six species of western Australian intertidal herbivorous gastropods and one chiton by an ashing technique. That portion of the feces not removed by ashing represented the non-organic feces fraction, composed primarily of calcium carbonate, the rock type upon which these molluscs lived. Expressed as percentages, the feces of the pulmonate limpet *Siphonaria kurracheenis* contained 69% carbonate, the feces of the acmaeid limpet *Callisella onychitis* contained 77% carbonate, the feces of *L. unifasciata* contained 73% carbonate, the feces of *N. australis* contained 80% carbonate, the feces of the nerite *Nerita atramentosa* contained 83% carbonate, the feces of the acmaeid limpet *Patelloida alticostata* contained 84% carbonate, and the feces of the chiton *Clavarizona hirtosa* contained 89% carbonate. Some of these species also possessed distinctly different radulae. For example, the cusp size of the *S. kurracheenis* radula was much smaller and that for *N. atramentosa* and much larger than other species with a similar body size. Black et al. (1988) concluded that such differences might influence competitive interactions of species occupying the same shore level, although they recognized that either specific behaviors or the nature of the rock surface might further mediate such interactions.

The vertical ranges of several of the Littorinidae of the Jamaican limestone platform overlap to a greater degree than some of the Australian species studied by Black et al. (1988), a situation that should only enhance the possibility of competitive interactions. There is little evidence, however, of such interactions among the Jamaican littorinids (Britton 1992, Lang et al. 1998, and this study). Furthermore, the nature of the radulae of the Jamaican species, although displaying some species-specific differences, are much more similar (Bandel & Kadolsky 1982) than those of the species studied by Black et al. (1988). The proportions of carbonate contained in the feces of both Australian and Jamaican intertidal molluscs, however, were very similar, an indication that both groups rely to a considerable degree on food attached to or within the substratum. It also possibly implies that such a food source is not especially limiting for the molluscs feeding upon it.

P. nucleus was found to have a very high fecal carbonate fraction (92.8%), consistent with expectations for individuals living in a high-energy, limestone boulder environment devoid of epilithic macrophytes or epiphytes. The mobile stones that dominate this beach are poorly suited for either algal attachment or growth. Likely food sources for *P. nucleus* include either diatoms washed up on the rocks by waves or endolithic algae, the latter necessitating ingestion of considerable rock carbonate while grazing for

TABLE 5.

Linear regressions predicting number of fecal pellets contained within rectum as a function of shell height (mm) for all species.

	Linear regression equation	Adjusted r^2
Planaxis nucleus	NP = 8.33 + (3.00 × SH)	0.039
Nodilittorina riisei	NP = −4.93 + (1.14 × SH)	0.058
N. ziczac	NP = −28.3 + (3.60 × SH)	0.336
N. angustior	NP = −10.8 + (2.03 × SH)	0.028
N. dilatata	NP = 43.4 − (1.63 × SH)	0.007
Tectarius antonii	NP = −14.2 + (2.61 × SH)	0.232
Cenchritis muricatus	NP = 14.7 + (1.13 × SH)	−0.002

Abbreviations: NP = number of pellets; SH = shell height.

TABLE 6.

Comparison of the fecal carbonate fraction expressed as a proportion among all species by a Kruskal-Wallis single-factor ANOVA by ranks ($\alpha = 0.05$; H = 201.7; df = 7; P = <0.001; * = significant difference) and among all species except *N. rissei* and *N. angustior* by a Kruskal-Wallis single factor ANOVA by ranks ($\alpha = 0.05$; H = 194.4; df = 5; P = <0.001; † = significant difference).

	Mean carbonate fraction	Standard deviation	CM-P	CM-R	NA	ND	NR	NZ	PN
C. muricatus on plants	0.186	0.133	—						
C. muricatus on rocks	0.748	0.057	*†	—					
N. angustior	0.670	0.142			—				
N. dilatata	0.883	0.043	*†	†		—			
N. riisei	0.887	0.058	*				—		
N. ziczac	0.749	0.049	*†			†		—	
P. nucleus	0.928	0.049	*†	*†	*			*†	—
T. antonii	0.931	0.040	*†	*†	*			*†	

Abbreviations as in Table 2, except CM-P, C. muricatus from plants; CM-R, C. muricatus from rocks.

this food source. Our data support the latter as a primary food source for *P. nucleus*. The only other planaxid for which diet has been studied is the Indo-Pacific *P. sulcatus*, which grazes upon epilithic algae but lives in a different habitat (i.e., bedrock shores where such algae are frequently abundant) (Rohde 1981).

The low-carbonate fraction in the fecal pellets of *C. muricatus* collected from plants (18.6%; Table 6) is likely the result of their most immediate diet prior to collection. If these individuals were grazing upon the plants or, more likely, on the epiphytic flora upon them, then the fecal carbonate fraction should be lower than that of conspecifics feeding exclusively upon the rocky substratum. The longer the duration of grazing upon plants, the greater the expected reduction of carbonate in the fecal pellets. In fact, *C. muricatus* from plants had the second highest standard deviation of all species tested (Table 6), indicating considerable variation among individuals, as should be expected. It has also been observed that *C. muricatus* moves readily to and from *R. americana* branches and the rocky platform (JCB, unpublished data). Thus, the carbonate present in the fecal pellets of specimens collected from plants is interpreted as carbonate ingested when the individual grazed upon limestone. Conversely, some *C. muricatus* individuals collected from rock could have a disproportionately low fecal carbonate content due to having recently grazed on the plants.

N. ziczac and *N. riisei* occupy approximately the same low shore habitat, characterized by frequent wave splash, occasional tidal inundation, and some epilithic and much endolithic algae on which to graze. The former is largest foreshore littorinid and produced the largest fecal pellets of those studied from this habitat (Tables 2 and 3). The latter, on the other hand, is the smallest species studied, but its fecal pellets were slightly larger and heavier than those of *N. angustior*, facilitated perhaps by a larger storage space within more globose shell. The feces of both species contained considerable carbonate, *N. ziczac* with a mean of 74.9% and *N. riisei* with a mean of 88.7%. The differences, although slight, might indicate a tendency toward resource partitioning, with *N. ziczac* perhaps taking more epilithic algae than *N. riisei*. On the other hand, these differences are not significant, perhaps due to the small number of pooled samples of *N. riisei*. The three pooled samples of *N. riisei* (Table 1) exhibited approximately the same variation as that for 38 samples for *N. ziczac* (SD of mean proportions = 0.058 and 0.049, respectively; Table 6).

The greatest variation among samples occurred with *N. angustior*, which also produced the smallest fecal pellets in terms of size (Table 2) and weight (Table 3), likely the result of a slender shell providing a small internal volume. This species was represented by only six pooled samples. Its feces contained a mean carbonate

TABLE 7.

Means and standard deviations of quantities of carbonate processed per individual based on an estimated 48-h defecation cycle and derived estimates of mean quantities of carbonate processed annually per individual and the total amount removed annually per species per m^2 on the Jamaican shore based on density estimates cited in Metz (1997).

	48-h defacation cycle		Derived estimates		
	Mean amount of carbonate processed/individual (mg)	Standard deviation	Mean amount removed/individual/y (g)	Mean density (n/m^2)	Total amount removed/y (g/m^2)
P. nucleus[1]	1.72	0.74	0.306	—	—
N. riisei	0.21	0.15	0.037	272	10.1
N. ziczac	1.19	0.94	0.211	80	16.9
N. angustior	0.23	0.15	0.040	480	19.2
N. dilatata	1.12	0.72	0.199	736	146.5
T. antonii	2.44	1.04	0.435	208	90.5
C. muricatus on plants	0.69	4.56	0.122	—	—
C. muricatus on rocks	3.07	1.91	0.546	128	69.9

[1] Densities unknown for these populations.

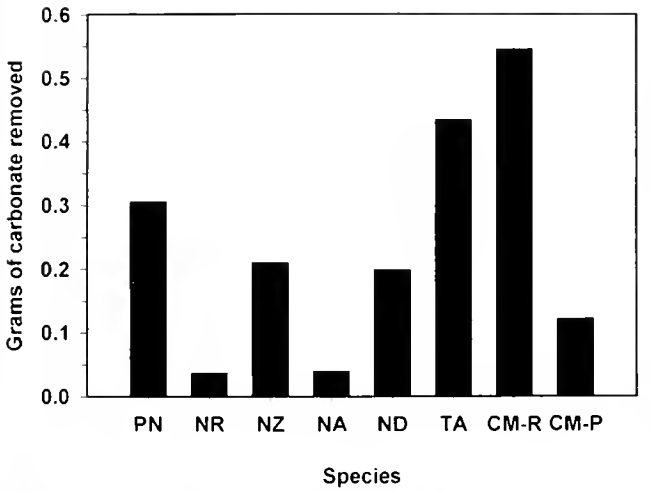

Figure 2. Estimated annual carbonate removed from the Jamaican limestone platform shore by individual littorinids, based on an estimated 48-h defecation cycle. Species arranged from low shore (left) to high shore (right). CM, *Cenchritis muricatus*; NA, *Nodilittorina angustior*; ND, *N. dilatata*; NR, *N. riisei*; NZ, *N. ziczac*; PN, *Planaxis nucleus*; TA, *Tectarius antonii*; CM-P, *C. muricatus* from plants; CM-R, *C. muricatus* from rocks.

fraction of 67.0%, with a standard deviation, expressed as a proportion, of 0.142 (Table 6). The range of *N. angustior* on the platform overlaps the frequently wetted upper distribution of both *N. ziczac* and *N. riisei* but also extends several meters landward into the lower range of *N. dilatata* where the rocks can remain unwetted by seawater for many days (Lang et al. 1998). One should expect the diet of *N. angustior*, of necessity, to include a considerable quantity of either endolithic algae or lichens, the former, at least, accompanied by a considerable quantity of carbonate. These samples, however, show that *N. angustior* fecal pellets possess the smallest proportion of carbonate of all species tested except for *C. muricatus* from plants. Because *N. angustior* is not known to graze upon maritime vegetation along this platform (JCB, personal observation), such behavior is not a potential cause for the lower carbonate content of the feces. If *N. angustior* focused upon the epilithic lichens, which are abundant within its range, fecal carbonate might be somewhat diminished. Such a diet might also suggest possible specialization in the radular structure, but Bandel and Kadolsky (1982) found the radulae of *N. ziczac*, *N. angustior*, *N. dilatata*, and *N. riisei* to be very similar. It seems unlikely, therefore, that *N. angustior* has such a specialized diet, but whether it does or does not is simply conjecture and requires further testing.

When all species were compared, the Kruskal-Wallis single factor ANOVA by ranks showed no significant differences among the four species with the highest fecal carbonate fraction (93.1–88.3%; *T. antonii*, *P. nucleus*, *N. riisei*, and *N. dilatata*; Table 6). There were no significant differences found among *N. riisei*, *N. dilatata*, *N. ziczac*, *N. angustior*, and *C. muricatus* on rocks (88.7–74.8%), but there were significant differences between *C. muricatus* on plants (18.6%) and all other species except *N. angustior* (67.0%). There were also significant differences within *C. muricatus* on plants (18.6%), those on rocks (74.8%), and *P. nucleus* (92.8%); and between *C. muricatus* from plants, those from rocks, and *T. antonii* (93.1%).

When *N. riisei* and *N. angustior* data were removed and the ANOVA was performed again without them, all of the significant differences previously detected remained unchanged, and two new significant differences appeared: one between *N. dilatata* and *C. muricatus* from rocks and the other between *N. dilatata* and *N. ziczac* (Table 6). Apparently, the large variation within the *N. angustior* samples obscured these differences when all eight groups were compared. In contrast, the large variation within the *C. muricatus* samples from plants did not alter the ANOVA results, probably because the mean proportion of carbonate was so much lower than the others that the high variation did not obscure any other significant differences.

Our original hypothesis, that fecal carbonate should vary with position on shore and increase toward the landward portion of the platform, was poorly supported by the data. Another pattern emerged, albeit unexpected and subtle, which might also be indicative of resource partitioning (Fig. 4). Depending upon shore position three pairs of littorinid associates are present, one of each pair having a relatively high fecal carbonate content and the other having somewhat to significantly less. On the high shore, *T. antonii* had the greatest amount of fecal carbonate, whereas rock-dwelling *C. muricatus* had the sixth highest amount and significantly less than that of *T. antonii* (Table 6). It is apparent from this study that *C. muricatus*, unlike *T. antonii*, feeds both upon endolithic algae and the epiphytic biota clinging to certain maritime plants, hence mediating the carbonate fraction of its feces. On the midshore, *N. dilatata* had the fourth greatest fecal carbonate fraction, and *N. angustior* had the seventh, a nonsignificant but distinctive difference. On the low shore, *N. riisei* had the third greatest fecal carbonate fraction, and *N. ziczac* had the fifth greatest, again a nonsignificant but distinctive difference. Perhaps the species pairs at the lower shore levels focus on somewhat different food sources, as does the high-shore pair. Alternately, and perhaps more parsimonious with these data, either food may be nonlimiting throughout the platform habitat, such that neither competition nor

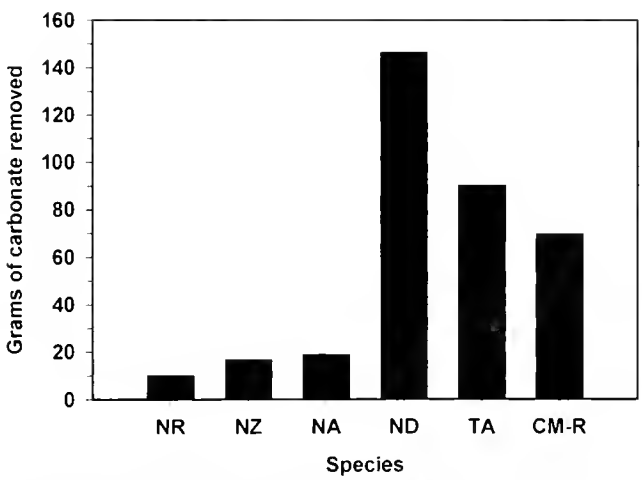

Figure 3. Estimated annual carbonate removed per m^2 from the Jamaican limestone platform shore by littorinid species, based on density estimates from Metz (1997). No density values were available for either *Planaxis nucleus* or *Cenchritis muricatus* from plants. Species arranged from low shore (left) to high shore (right). CM, *Cenchritis muricatus*; NA, *Nadilittorina angustior*; ND, *N. dilatata*; NR, *N. riisei*; NZ, *N. ziczac*; PN, *Planaxis nucleus*; TA, *Tectarius antonii*; CM-P, *C. muricatus* from plants; CM-R, *C. muricatus* from rocks.

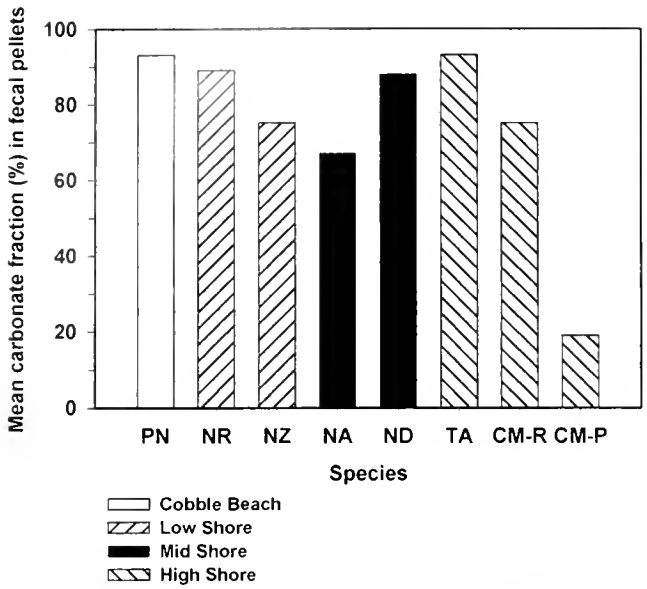

Figure 4. Mean carbonate fraction in fecal pellets with species grouped according to habitat from low shore (left) to high shore (right). CM, *Cenchritis muricatus*; NA, *Nodilittorina angustior*; ND, *N. dilatata*; NR, *N. riisei*; NZ, *N. ziczac*; PN, *Planaxis nucleus*; TA, *Tectarius antonii*; CM-P, *C. muricatus* from plants; CM-R, *C. muricatus* from rocks.

resource partitioning is an issue or each species is broadly opportunistic with respect to food selection, grazing on epilithic primary production when available and on endolithic primary production when epiphytes are reduced in abundance.

The platform littorinids have a significant bioerosive impact on this tropical limestone shore. The combined grazing of *C. muricatus* and *T. antonii* strips considerable carbonate (160.4 g/m^2/y) from the upper shore. This translates to the removal of about 1,472 kg annually along the approximately 500-m-long platform and across an upper shore band about 24 m wide. Although *N. dilatata* is a moderate-sized species with each individual having only a modest bioerosive impact, it also has the highest population density (a mean of 736 individuals/m^2). Thus, it significantly impacts the central platform, removing limestone at the rate of 146.5 g/m^2/y (Table 7; Fig. 3), and, because its range covers a band about 17 m wide, it removes a little more than 1,245 kg annually from this shore. The lower shore, although having more littorinid species, experiences the least amount of littorinid bioerosion, both per species and in total (Table 7). Several factors contribute to this diminished impact. The lower shore is horizontally limited, being 7 m wide at best and hardly more than 5 m wide if the range of *N. angustior* is excluded. Population densities of *N. ziczac* are very low, minimizing its impact. Although both *N. angustior* and *N. riisei* can be very abundant locally, dense populations are patchy and often separated by areas of sparse density. Their small size also reduces the bioerosive impact of each individual. Finally, the presence of epilithic algae on some parts of the lower shore may provide an almost carbonate-free alternate food source. Thus, the total annual carbonate removal rate, combining the grazing impact of *N. riisei*, *N. ziczac*, and *N. angustior*, is 46.2 g/m^2/y, or almost 140 kg annually. It should be noted, however, that other, much larger, grazing gastropods, especially *Nerita tesselata*, *N. peloronta*, and *N. versicolor*, and one common chiton, *Acanthopleura granulata*, occupy the lower shore (Minton & Gochfeld 2001) and have the potential to remove considerably greater quantities of carbonate than the low shore littorinids. For the entire platform, all littorinid species account for the removal of a little more than 2,850 kg of limestone annually.

In conclusion, all species studied ingested considerable quantities of carbonate, probably indicating that all rely to some degree upon endolithic algae for food. *C. muricatus* was the only species clearly identified as also having an alternate food source, probably epiphytes on the branches, stems, and leaves of the maritime shrub *R. americana*. Although the rock-dwelling Littorinidae ingested different quantities of carbonate, ranging from 67–93%, there was no clear relationship between the amount of carbonate ingested and the position each species occupied on shore. However, species pairs that cohabitate on particular shore levels possessed noticeably different mean fecal carbonate fractions, leaving open the possibility that some form of resource partitioning might be involved. There was no indication, however, what it might be. The quantity of carbonate in *P. nucleus* fecal pellets was similar to that found in the several littorinid species studied, but the size and morphology of *P. nucleus* fecal pellets (spindle shaped) were significantly different from those found among the Jamaican Littorinidae (ovoid shaped). The Littorinidae occupying the rocky intertidal platform remove significant quantities of carbonate from the shore each year and should be considered important bioerosive species.

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ELLOBIIDAE—LOST BETWEEN LAND AND SEA

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ABSTRACT The Ellobiidae are a diverse group of archaeopulmonate snails living mostly near the sea. Their shell length ranges from barely 2 mm to over 100 mm. Six structural types of reproductive system set the boundaries for the subfamilial division: Pythiinae, Ellobiinae, Carychiinae, Cassidulinae, Pedipedinae, and Melampodinae. This division is in general supported by four structural types of nervous system and by the internal morphology of the penial complex. The halophile ellobiids are commonly associated with the supratidal fringe of mangroves and salt marshes; they are also important components of the supratidal biota of the mobile rocky shore. The various species occupy different portions of the shore. In rocky habitats, in addition to horizontal zonation, the ellobiids also partition their vertical distribution. The purpose of this study is to describe the zonation patterns of the halophile ellobiids of the cobble shores of the Açores and, in less detail, of the associated malacofauna.

KEY WORDS: Ellobiidae, systematics, morphology, anatomy, ecology, cobble shores, Açores

INTRODUCTION

The Ellobiidae have long been recognized as a cohesive taxonomic unit (Lamarck 1809) and were illustrated in the most important iconographic works of the nineteenth century (Reeve 1841, Reeve 1877, Küster 1844, Kobelt 1897–1901). Classification was based exclusively or primarily on shell morphology. Pfeiffer (1856, 1876), elaborating on shell descriptions of all species known at the time and adding many of his own, provided an important monographic treatment for the group. Zilch's (1959) subfamilial arrangement was also based on shell characteristics. Radular (Odhner 1925) and anatomical features have since been included in taxonomic research and have contributed greatly to the clarification of the relationships of the various taxa (Morton 1955, Martins 1996a, Martins 1996b, Martins 1998).

Ellobiids are important members of the supratidal communities of marshes and mangroves, and their contribution to the ecological balance of those environments has been somewhat recognized in studies dealing with their general biota (Berry 1963, Brown 1971, Sasekumar 1974, Murty & Balaparameswara Rao 1977, Li & Gao 1985). More specific studies have been done on *Melampus bidentatus* and *M. coffeus* (Hausman 1932, Holle & Dineen 1957, Morrison 1958, Morrison 1959, Golley 1960, Grandy 1972, Russell-Hunter et al. 1972, Orton 1976).

The various species of halophile ellobiids occupy different portions of the shore, their distribution being loosely related to their

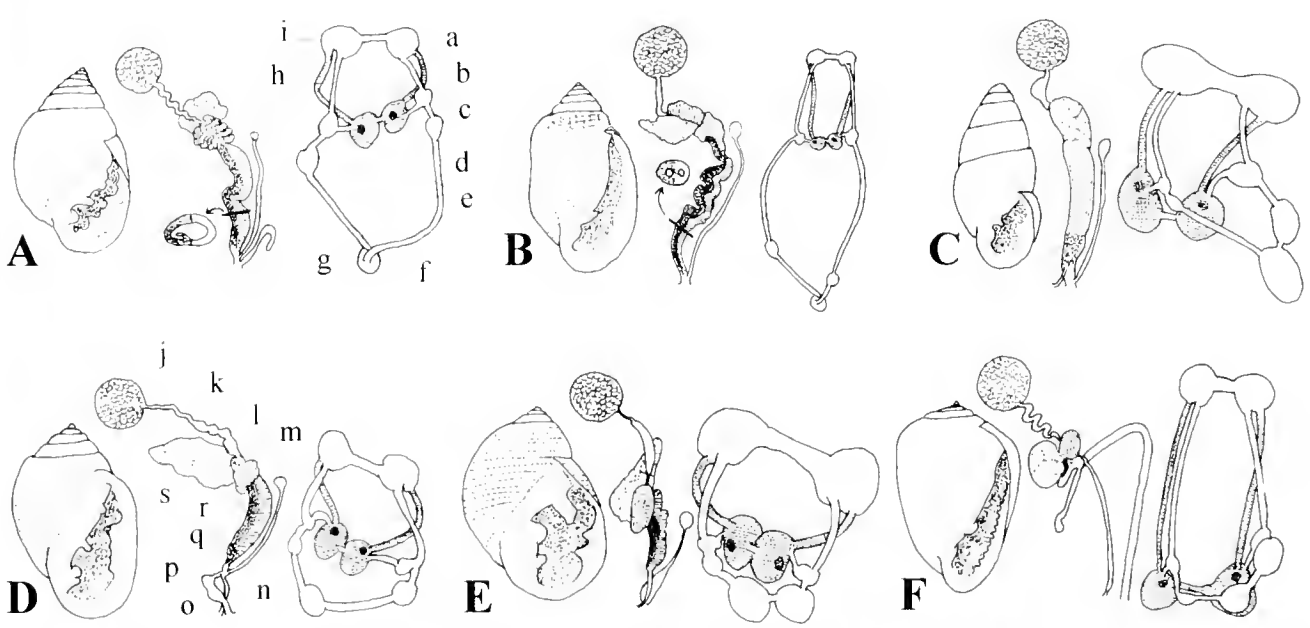

Figure 1. Shell, reproductive system, and nervous system of selected species of Ellobiidae, exemplifying the structural patterns characterizing the various subfamilies. A, Pythiinae *Pythia scarabaeus* (L., 1758); B, Ellobiinae *Ellobium aurismidae* (L., 1758); C, Carychiinae *Carychium minimum* (O.F. Müller, 1774); D, Cassidulinae *Cassidula aurisfelis* (Bruguière, 1789); E, Pedipedinae *Pedipes pedipes* (Bruguière, 1791); F, Melampodinae *Melampus coffeus* (L., 1758). a, cerebro-pedal connective; b, pleural ganglion; c, pleuro-parietal connective; d, parietal ganglion; e, pedal ganglion; f, parieto-visceral connective; g, visceral ganglion; h, cerebro-pleural connective; i, cerebral ganglion; j, ovotestis; k, hermaphroditic duct; l, posterior mucous gland; m, bursa; n, bursa duct; o, posterior vas deferens; p, vagina; q, prostate gland; r, anterior mucous gland; s, albumen gland. (Adapted from Martins 1996a.)

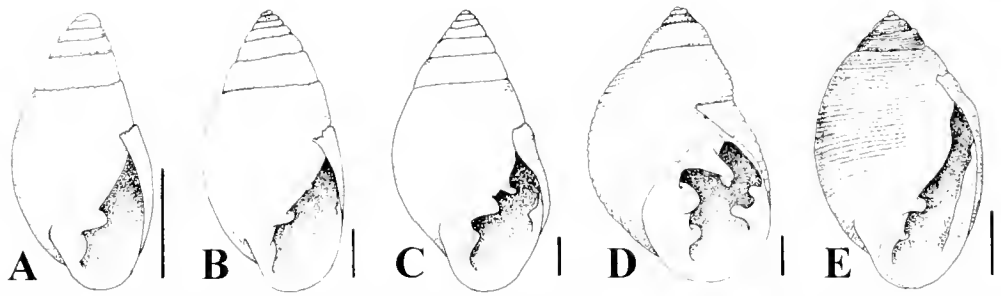

Figure 2. Ellobiids of the cobble shores of the Açores. A, *Auriculinella bidentata*; B, *Myosotella myosotis*; C, *Ovatella vulcani*; D, *Pedipes pedipes*; E, *Pseudomelampus exiguus*. Scale bars = 1 mm.

taxonomic affiliation. The Pythiinae (*Pythia* and, to a lesser extent, *Myosotella*) venture farther inland and may live in an almost terrestrial environment, whereas the Pedipedinae prefer the upper intertidal. Within the Melampodinae, some species of *Melampus* are found usually in mangroves and marshes, whereas others and *Tralia* prefer cobble shores.

The ellobiids abound also on cobble shores, and attempts have been made to quantify their horizontal and vertical distributions on such environments (Martins 1980, Martins & Cunha 1992). Morton et al. (1998) summarized the ecology of ellobiids on the cobble shores of the Açores.

This paper will briefly introduce the Ellobiidae as a morphologically and anatomically highly diverse family and, reinterpreting data from Martins (1980), present a model for their distribution along the cobble shores of the Açores.

MATERIALS AND METHODS

Morphology and Anatomy

The ellobiids range in shell size from barely 2 mm (*Leuconopsis*) to 100 mm (*Ellobium*); most species, however, do not exceed 25 mm. The smallest taxa inhabit moderately exposed rocky shores, whereas the largest species prefer the still backwaters of mangrove swamps.

Currently, six subfamilies of the Ellobiidae are recognized: Pythiinae, Ellobiinae, Carychiinae, Cassidulinae, Pedipedinae, and Melampodinae. Although shell morphology and internal anatomy have been considered in delimiting the subfamilies, these are based primarily on characteristics of the reproductive and nervous systems (Morton 1955, Martins 1996a, Martins 1996b, Martins 1998).

The dentate appearance of the shell aperture establishes a generalized ellobiid profile. However, the elaboration of the apertural teeth is far from uniform and, although relatively constant at the genus level, shows variability within and similarities across the subfamilies, which is not consistent with the corresponding internal anatomy. For example, a tridentate inner lip with the middle tooth strongest is seen in *Myosotella* (Pythiinae), *Ellobium* (Ellobiinae), and *Tralia* (Melampodinae); a narrow, elongated aperture, typical of *Melampus*, is also found in *Microtralia* (Cassidulinae) and *Pseudomelampus* (Pedipedinae); a double columellar tooth, typical of *Pedipes*, is also found in some *Auriculastra* (Ellobiinae) as well as in *Creedonia* (Cassidulinae).

The anatomy of the reproductive and nervous systems shows a series of patterns that lead to more consistent assemblages (Fig. 1) (see also Martins 1996a, Martins 1998). The subfamilies can then be characterized as follows: (1) Pythiinae: monaulic, entirely glandular pallial gonoducts and long visceral nerve ring (pleural, parietal and visceral ganglia and respective connectives) with both parieto-visceral connectives of about the same length; (2) Ellobiinae: diaulic, entirely glandular pallial gonoducts and long visceral nerve ring with the right parieto-visceral connective much shorter than its left counterpart; (3) Carychiinae: monaulic, entirely glandular pallial gonoducts, the prostate gland anterior to the mucous gland, whereas in all other subfamilies both glands are located side-by-side; nervous system as in the Ellobiinae; (4) Cassidulinae: monaulic, entirely glandular pallial gonoducts, the distal portion of the bursa duct transformed into a nonglandular, well-developed vaginal atrium where the oviduct empties; nervous system with visceral and esophageal (cerebral and pedal ganglia, cerebro-pedal

TABLE 1.

Species of mollusks mentioned in the text and abbreviations used in Table 2.

Species	Abbreviation
Halophile pulmonates (ellobiids)	
Auriculinella bidentata (Montagu, 1808)	Ab
Myosotella myosotis (Draparnaud, 1801)	Mn
Ovatella vulcani (Morelet, 1860)	Ov
Pedipes pedipes (Bruguière, 1789)	Pp
Pseudomelampus exiguus (Lowe, 1832)	Pe
Terrestrial pulmonates	
Euconulus fulvus (O. F. Müller, 1774)	Ef
Lauria anconostoma (Lowe, 1831)	La
Leiostyla fuscidula (Morelet, 1860)	Lf
Oestophora barbula (Rossmässler, 1838)	Ob
Oxychilus draparnaudi (Beck, 1837)	Od
Punctum azoricum (de Winter, 1988)	Pa
Systellomatophoran	
Onchidella celtica (Cuvier, 1817)	Oc
Prosobranchs	
Alvania mediolittoralis Gofas, 1989	Am
Assiminea eliae Paladilhe, 1875	Ae
Cingula trifasciata (Adams, 1798)	Ct
Fossarus ambiguus (Linnaeus, 1758)	Fa
Littorina striata King & Broderip, 1832	Ls
Manzonia unifasciata (Dautzenberg, 1889)	Mu
Melaraphe neritoides (Linnaeus, 1758)	Mn
Paludinella littorina (delle Chiage, 1828)	Pl
Patella gomesii Drouët, 1858	Pg
Peringiella ovummuscae (Gofas, 1990)	Po
Thais haemastoma (Linnaeus, 1767)	Ts
Bivalves	
Cardita calyculata Linnaeus, 1758	Cc
Lasaea adansoni (Gmelin, 1791)	Lr
Mysella bidentata (Montagu, 1803)	Mb

TABLE 2.

Number of specimens of ellobiids and presence (x) of associated malacofauna per m^2 along transects on five cobble shores of the Açores.

Species	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	0	−1	−2	−3	−4
Transect A																					
Mn	6	8	2	12	4	6	9	2	–	–	3	–	8	–	–	–	–	–	–	–	
Ov	–	–	–	–	–	–	–	–	–	–	3	4	22	23	–	105	105	60	40	1	
Pe	–	–	–	–	–	–	–	–	–	–	3	1	75	290	10	190	600	250	50	2	
Pp	–	–	–	–	–	–	–	–	–	–	1	–	16	50	–	170	600	55	75	13	
Ab	–	–	–	–	–	–	–	–	–	–	–	–	3	2	–	15	63	23	7	2	
Pa	x	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
Ef	x	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
Lf	x	x	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
La	x	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
Ae	–	–	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
Mn	–	–	–	–	–	–	–	–	x	–	–	–	x	x	x	x	x	x	x	x	
Ls	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	x	x	x	
Ts	–	–	–	–	–	–	–	–	x	–	–	x	x	–	–	–	x	–	–	–	
Pl	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	x	–	–	
Ct	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	x	x	
Fa	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	x	x	–	
Po	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	x	x	x	
Pg	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	x	
Transect B																					
Mn	–	–	–	–	–	–	–	17	10	70	70	80	90	130	20	–	–	–	–	–	
Ov	–	–	–	–	–	–	–	3	–	–	1	10	13	140	1,200	4,850	2,680	590	65	1	
Pe	–	–	–	–	–	–	–	–	–	–	2	2	1	30	430	470	–	–	–	–	
Pp	–	–	–	–	–	–	–	–	–	–	–	1	2	10	270	950	1,100	320	35	1	
Ab	–	–	–	–	–	–	–	–	–	–	–	–	–	5	75	50	60	70	12	1	
La	–	–	–	–	–	–	–	x	–	–	–	–	–	–	–	–	–	–	–	–	
Ob	–	–	–	–	–	–	–	x	–	–	–	–	–	–	–	–	–	–	–	–	
Od	–	–	–	–	–	–	–	x	–	–	–	–	–	–	–	–	–	–	–	–	
Ae	–	–	–	–	–	–	–	x	x	x	x	x	x	x	x	x	x	x	x	x	
Mn	–	–	–	–	–	–	–	–	–	–	–	–	x	x	x	x	x	x	x	x	
Ls	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	x	x	x	
Pl	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	x	x	x	x	
Ct	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	x	x	x	x	
Fa	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	x	x	x	
Pg	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	x	x	
Th	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	–	
Transect C																					
Mm	–	–	–	–	–	–	–	270	550	440	110	170	23	5	–	–	–	–	–	–	–
Ov	–	–	–	–	–	–	–	1	5	6	12	5	10	5	1	15	2	–	1	–	–
Pe	–	–	–	–	–	–	–	–	120	1,140	120	700	720	210	15	20	2	2	2	3	1
Pp	–	–	–	–	–	–	–	–	–	–	–	25	85	75	3	20	6	–	1	2	–
Mn	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	x	x	x	–	–
Ls	–	–	–	–	–	–	–	–	–	–	–	x	x	x	x	x	–	–	–	–	–
Ct	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	x	x	–
Fa	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	–	x	x	–
Po	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	x	–	x	–
Pg	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	–
Oc	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	x	–
Transect D																					
Mm	–	–	–	–	15	10	15	25	15	57	30	45	75	41	42	45	7	11	2	–	–
Ov	–	–	–	–	–	–	–	–	–	–	–	–	1	–	3	26	80	140	95	3	–
Pe	–	–	–	–	–	–	–	15	33	5	5	21	25	100	65	50	20	25	–	–	–
Pp	–	–	–	–	–	–	–	–	1	–	–	–	7	31	106	105	85	80	1	5	–
Mn	–	–	–	–	–	–	x	x	x	x	x	x	x	x	x	x	x	x	x	x	–
Ls	–	–	–	–	–	–	–	–	–	x	–	–	x	x	x	x	x	x	x	x	–
Pl	–	–	–	–	–	–	–	–	–	–	–	–	–	x	x	x	x	x	x	–	–
Fa	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	x	x	x	–
Pg	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	–

Continued

TABLE 2.

continued

Species	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	0	−1	−2	−3	−4
Transect E																					
Mn	–	–	–	–	20	40	15	30	70	85	40	72	15	–	–	–	–	–	–	–	
Ov	–	–	–	–	–	–	–	–	–	2	5	23	400	255	515	500	15	–	–	–	
Pe	–	–	–	–	–	–	–	–	2	47	80	700	1,730	1,860	1,440	200	1	–	–	–	
Pp	–	–	–	–	–	–	–	–	–	–	–	60	485	1,250	1,760	1,080	1,000	60	4	1	
Ab	–	–	–	–	–	–	–	–	–	–	–	–	–	2	5	2	–	–	–	–	
Ob	–	–	–	–	–	x	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
Mn	–	–	–	–	–	–	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
Ls	–	–	–	–	–	–	x	–	–	–	x	x	x	x	x	x	x	x	x	x	
Pl	–	–	–	–	–	–	–	–	–	x	x	x	x	x	x	x	x	x	x	x	
Ct	–	–	–	–	–	–	–	–	–	–	–	x	x	x	x	x	x	x	x	x	
Mu	–	–	–	–	–	–	–	–	–	–	–	x	x	–	–	–	–	–	–	–	
Fa	–	–	–	–	–	–	–	–	–	–	–	–	x	x	x	x	x	x	x	x	
Am	–	–	–	–	–	–	–	–	–	–	–	–	–	x	–	x	x	x	x	x	
Lr	–	–	–	–	–	–	–	–	–	–	–	–	–	x	–	x	x	x	x	x	
Mb	–	–	–	–	–	–	–	–	–	–	–	–	–	x	–	–	–	–	–	–	
Cc	–	–	–	–	–	–	–	–	–	–	–	–	–	x	–	x	x	–	–	–	
Pg	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	x	x	x	x	x	

High tide level at 0; remaining numbers are meters away from 0, with negative numbers being seaward. See text for transect identification and Table 1 for species abbreviations (data from Martins 1980).

connectives and pedal commissure) rings moderately wide, approximately of equal size; (5) Pedipedinae: monaulic to incipient semidiaulic, pallial gonoducts with glandular coverage only on the proximal (posterior) half; visceral nerve ring extremely concentrated, right connectives of the esophageal nerve ring shorter than their left counterparts; and (6) Melampodinae: advanced semidiaulic pallial gonoducts, the residual glandular portion concentrated proximally (posteriorly); visceral nerve ring extremely concentrated, connectives of the esophageal nerve ring of approximately equal length.

Additional diagnostic features have been sought in the internal morphology of the penis, which have in many cases corroborated the assemblages just presented (Martins 1996a, Martins 1998).

Ellobiids on Cobble Shores

The ellobiids are common inhabitants of the supratidal of certain rocky habitats. Cobble beaches are a common feature of the Açorean rocky shore (Morton et al. 1998) and are the habitat of five species of halophile ellobiids (Fig. 2). The distribution of these species along the cobble shores has previously been recorded (Martins 1980). Following is an elaboration on those data to present a model for their distribution. The distribution of the associated malacofauna will also be presented (Table 1).

Five of the original 11 transects of 1-m^2 quadrats (Martins 1980) were selected on the basis of their relatively similar length to provide a more or less homogeneous set of data (Table 2). The locations of the transects and the sampling dates are as follows:

Transect A: West of Ponta do Alcaide, Silveira, Terceira, Açores. February, 1976.

Transect B: Calhau da Pata, Caminho de Baixo, São Mateus, Terceira, Açores. March, 1976.

Transect C: Pópulo, São Miguel, Açores. January, 1976.

Transect D: Ponta de São Pedro, Vila Franca do Campo, São Miguel, Açores. February, 1976.

Transect E: Atalhada, Lagoa, São Miguel, Açores. March, 1976.

Cobble shores are, in principle, exposed to at least moderate wave action. All but transect C have moderate to high exposure to wave action, with transect C being more protected.

Kite diagrams were constructed to illustrate the distributions of the species. For ellobiids, data (as percentages) from the five transects were combined, and the percentages present at each height above or below high tide level were used to construct the kite diagrams. For other species, presence/absence was simply totaled for all five quadrats at a particular height, for a maximum of five.

RESULTS

The distribution of the ellobiids and associated malacofauna along the cobble shores of the Açores is summarized in Figure 3.

DISCUSSION

The five species of ellobiids exhibit a definite pattern of distribution along the cobble shore. *Myosotella myosotis* lives highest up, almost assuming a terrestrial habitat, indicated by the presence of terrestrial pulmonates at these levels. It is followed by *Ovatella vulcani* and *Pseudomelampus exiguus*, which closely overlap. Additional information (Martins 1980) showed that, although overlapping along the transect, both species are somewhat separated vertically in the cobble accumulation. The former prefers the upper levels, whereas the latter is more abundant toward the bottom, frequently found under half-buried porous rocks. *Pedipes pedipes*, although overlapping with the latter two species, aggregates closer to a level just above the high tide level, a distribution more narrowly exhibited by *Auriculinella bidentata*. Here, too, there appears to exist vertical zonation, with *P. pedipes* preferring the upper levels, whereas *A. bidentata* lives underneath the rocks (Martins 1980). The single specimen of *P. pedipes* recorded from D9 (quadrat 9 of transect D) could be interpreted as accidental presence because the next closest recording is another single specimen in A6. However, transect D is in a highly exposed area, and

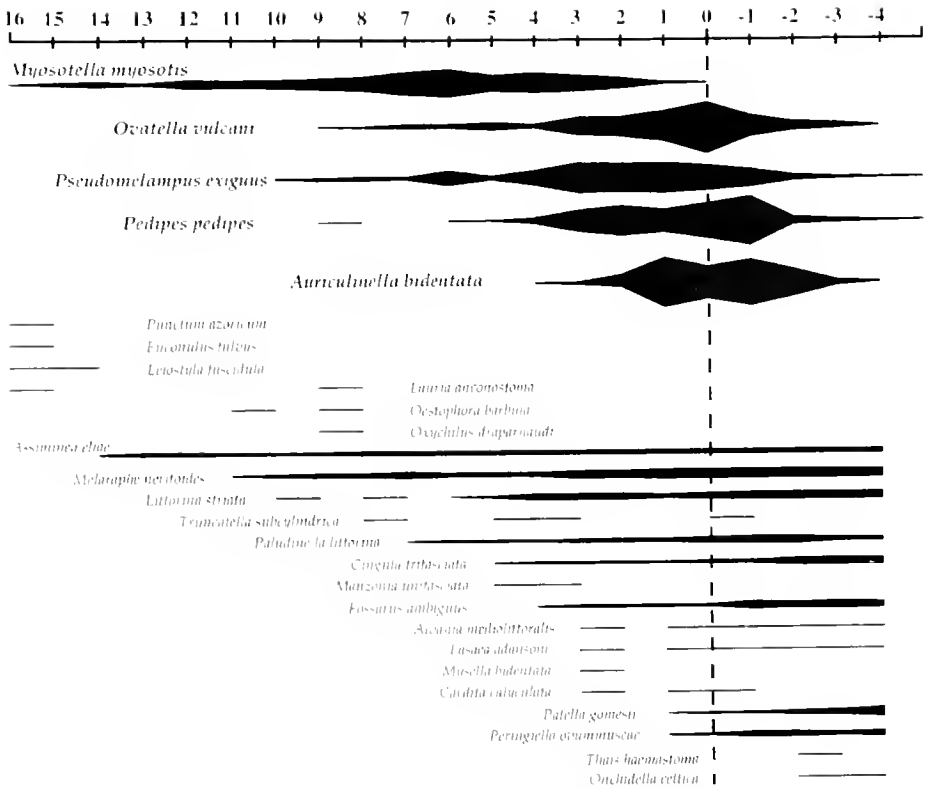

Figure 3. Distribution of ellobiids and associated malacofauna along the cobble shores in the Açores. Combined percentage (ellobiids) and cumulative presence (associated malacofauna) on five transects of different lengths. Dotted line (0) marks the high tide limit, located at the landward end of quadrat 0. Associated malacofauna not recorded in quadrat −4. (Adapted from Martins 1980.)

strong hydrodynamism could explain this apparently unusual presence, an interpretation supported by the equally more landward distribution of *Pseudomelampus exiguus* in transect D.

The ellobiids of a boulder shore of Hong Kong, although a different species assemblage, exhibited similar distribution patterns (Martins & Cunha 1992). Their horizontal distribution was more clearly understood when their vertical distribution in each quadrat along the transect was examined: In general, the horizontally widely distributed species maintained some preference for an upper vertical level as they approached the sea. The data for the Açores, presented here, lack transect profiles and vertical quantification, not allowing for inferences of this kind. However, as already referred to by Martins (1980), in many instances similar behavior occurs in the Açorean species.

Worth mentioning, within the associated malacofauna, is the wide horizontal range of *Assiminea eliae*, restricted to Terceira Island, contrasting with the more marine preference of another assimineid, *Paludinella littorina*, which is present on both islands.

The two littorinids present in the studied areas exhibit their commonly observed relative distribution pattern, with *Melaraphe neritoides* extending farther inland than *Littorina striata* (Morton et al. 1998).

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WHEN TO MOVE AND WHERE TO GO: MOVEMENT BEHAVIOR OF THE TROPICAL LITTORINID *CENCHRITIS MURICATUS* (LINNAEUS, 1758)

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ABSTRACT Movement behavior in intertidal molluscs is related to the intensity of physical and biological stress. Tropical littorinids are exposed to extreme environmental conditions, and movement at night or on a tidal cycle may alleviate desiccation and heat stress, as would returning to a sheltered location following foraging. We examined the movement behavior of individually marked *Cenchritis muricatus* on a Jamaican shore for 30 days. On 6 days, selected from different parts of the lunar cycle, snail locations were monitored at 3-h intervals. Movement did not occur on a diurnal, tidal, or lunar cycle. Although *C. muricatus* preferentially rested in crevices, there was no evidence of homing behavior. Snails resting on exposed rock surfaces were four times more likely to move than snails resting in more sheltered microhabitats. In general, movement in *C. muricatus* appears to occur in direct response to wetting: >89% of movements occurred within 12 h after rainfall or heavy dew. We believe this behavior is primarily a response to desiccation stress, but it may also facilitate foraging.

KEY WORDS: movement behavior, movement periodicity, microhabitat, tropical littorinid

INTRODUCTION

Rocky shore intertidal gastropods display a variety of movement behaviors, ranging from homing to a specific location on the rock to periodic migrations up and down the shoreline. Homing to a particular scar on the rock following foraging excursions is well known in limpets (Underwood 1979, Branch 1981, Chelazzi et al. 1988, Della Santini et al. 1995) and chitons (Chelazzi et al. 1988, Little 1989). Vertical migrations or periodicity in foraging activity occurring on a diurnal or tidal cycle have been demonstrated in neritids (Underwood 1979, Little 1989), limpets (Hartnoll & Wright 1977, Branch 1981, Little 1989, Gray & Naylor 1996), and littorinids (Little 1989, Ohgaki 1989, Norton et al. 1990). In addition, seasonal migrations, often associated with reproductive events, have been observed in some limpets (Underwood 1979, Chelazzi et al. 1988, Della Santini et al. 1995) and littorinids (Chelazzi et al. 1988, Takada 1992).

Most studies of movement behavior in littoral gastropods assume that desiccation stress is a primary influence on the evolution of the timing of movement (Little 1989). For gastropods occupying tropical rocky shores, which are exposed to direct sunlight and extremely high temperatures, individuals should move when the risk of desiccation and heat stress is lowest, such as during rain or high tides or during the night when temperatures are cooler. Tropical high-shore gastropods may avoid potentially lethal daytime conditions by use of a limited cyclic activity period and by selective use of microhabitat (Garrity & Levings 1984) as well as by withdrawing into their shells and cementing the shell to the rock with a mucus holdfast (McMahon & Britton 1991). Supralittoral gastropods also display a variety of morphological and physiological adaptations, which enable them to persist under extreme conditions of heat and desiccation (McMahon 1991, Britton 1992).

The Caribbean littorinid *Cenchritis muricatus* (Linnaeus, 1758) occupies the highest position in the supralittoral fringe of rocky shorelines (Fraenkel 1968, Lang et al. 1998, Minton & Gochfeld, 2001). The zone occupied by *C. muricatus* is subjected to extreme environmental conditions of heat and desiccation and is rarely inundated by seawater. Few studies have examined the ecology of *C. muricatus* (Lang et al. 1998), and little is known about its behavior patterns, although Lang et al. (1998) noted that this species spends most of its time in repose, and Kaplan (1988) suggested that *C. muricatus* must migrate downshore to forage at night when it is cooler. The objectives of this study were to investigate the movement behavior of *C. muricatus* over a range of temporal scales. Unlike many earlier studies, the present study spans an entire lunar cycle to discriminate between movement patterns based on diurnal, tidal, or lunar cues.

MATERIALS AND METHODS

This study was carried out on an uplifted Holocene limestone reef on the north coast of Jamaica near the Hofstra University Marine Laboratory at Priory, St. Ann. This north-facing platform rises up to 3 m above sea level and ranges from 10–25 m wide in the study area. The substrate is predominantly unvegetated and heavily microkarstified. Snails were selected from ten sites within an area 100 m long.

On 23 July 1998, five adult individuals of *C. muricatus* (length: 17.1 ± 0.6 mm; width: 13.2 ± 0.7 mm) were selected from each of ten sites, marked, and returned to their original locations. Snails were marked using colored nail polish or enamel paint, followed by an application of clear enamel to prevent the color from wearing off. Snails from the same site were painted different colors so that we could identify individuals.

After 3 days we found as many of the marked snails as we could and placed a numbered coin on the rock beside them to denote their present location. For each snail, we measured the nearest distance and compass heading to the ocean and the microhabitat in which it was located. Distance measurements were determined using a fiberglass measuring tape. Distances recorded in this study represent the shortest distance measured between the snail's previous and present locations. Because it is unlikely that snails travel in straight lines, these values represent the minimum distances traveled and are therefore underestimates of the rate of movement. We used four microhabitat classifications: surface (on the exposed rock surface), pit (depression in the rock of sufficient size to house only a single adult individual), crevice (depression in

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the rock of sufficient size to house more than one adult individual), and vegetation (either on or under vegetation). In addition, a dab of paint corresponding to the color of the snail was placed on the rock at the snail's initial location to determine whether the snail showed evidence of homing to this site.

During subsequent observations, the following data were collected: distance moved from the previous location (denoted by the coin), compass direction moved, and microhabitat of the new location. The coin was then moved to the snail's new location.

Observations were made daily, generally between 8 AM and 12 PM, from 26 July to 24 August 1998, corresponding roughly to a lunar cycle. In addition, during 6 days randomly selected from within this period (27 July, 29 July, 4 August, 8 August, 19 August, and 22 August), observations were made at approximately 3-h intervals for 24 h. To test the hypothesis that movement in *C. muricatus* was restricted to nocturnal periods, a chi-square test was performed on a 2 × 2 contingency table that compared the number of nocturnal vs. diurnal observation periods during which movement vs. no movement was recorded. Tidal periodicity in movement behavior was analyzed by correlating the relative tide height, measured with an automated data logger based at the Hofstra University Marine Laboratory, with movement patterns for the dates on which 24-h observations were conducted. The tide data for 27–28 July were omitted from this analysis due to a malfunction of the data logger. Lunar periodicity in snail movement behavior was examined by converting the lunar phase into a relative value between +1 (full) and −1 (new) and correlating this value with the percentage of snails moving on each day over the lunar cycle. For this analysis, snails that were observed to move multiple times during the 24-h observation periods were included only once. Rainfall was monitored using a plastic rain gauge.

Microhabitat Availability

At each site, microhabitat availability was determined by haphazardly placing 0.0625-m^2 quadrats on unvegetated substrate in the zone in which *C. muricatus* occurred (n = 3–12 quadrats per site, depending on the width of this zone). The type of microhabitat (crevice, pit, or surface) present at each of 16 points within the quadrat was identified. All replicates (n = 63) were pooled to determine mean proportions of each microhabitat available to *C. muricatus*. The overall preference of *C. muricatus* for each microhabitat was determined by comparing the number of snails occupying each microhabitat with the availability of that microhabitat using a chi-square test. Additionally, for each snail, its occurrence in the three microhabitats was compared with the availability of each microhabitat by a chi-square test to investigate its apparent microhabitat preference. Although the number of individual snails associated with vegetation are reported, percent cover of vegetation was not quantified, and therefore the preference of snails for vegetation was not analyzed.

Wetting Experiment

To determine whether the quantity or salinity of wetting influenced the movement of *C. muricatus*, snails were exposed to different quantities of fresh water or to seawater. On a hot sunny day, 16 1-m^2 quadrats were haphazardly laid on a relatively flat area of shoreline in the general vicinity of, but not actually in, our study sites. Quadrats were randomly assigned to four treatments: no water (control), 250 ml fresh water, 500 ml fresh water, or 250 ml sea water. These volumes of water were equivalent to 0.25 mm and 0.5 mm of rainfall or wave splash. All *C. muricatus* within each quadrat were counted initially. Water was applied to the treatment quadrats using a plastic bottle with a piece of plastic wrap attached to the top with a rubber band. Several small holes were punched in the plastic wrap, which allowed the water to be sprinkled evenly over the substrate. Once the quadrats were wetted, we determined the percentage of snails that had moved after 10 min. Control quadrats were not wetted but were monitored in the same way. A one-way analysis of variance (ANOVA) was used to examine the effect of wetting on snail movement. Significant results were then analyzed using Tukey's pairwise comparisons with an overall $\alpha = 0.05$.

RESULTS

Altogether, 64 periods were sampled, and 37–46 marked snails (42.6 ± 2.47) were located during each observation period. We made 2,726 total observations on 48 different snails (56.7 ± 13.6 observations per snail).

Rates and Direction of Movement

The majority of our observations (91%) were of snails that did not move. When *C. muricatus* did move, they were able to move relatively rapidly. The maximum rate of movement recorded was 38 cm/h, with a mean overall rate of movement of 5.0 ± 5.5 cm/h (n = 175). Individuals of *C. muricatus* did not display any consistent preferred direction of movement (Bonferroni-corrected Rayleigh's test for random angular dispersion, $\alpha = 0.001$; maximum $R = 5.9$; $n = 48$ snails; minimum $P = 0.005$). When the movements of all snails were pooled and the direction of the ocean was standardized, there was no evidence of directional movement (Rayleigh's test, $R = 29.5$; $n = 366$ observations; $P > 0.05$) (Fig. 1).

We found no evidence of homing in *C. muricatus*, either to the initial marked "homes" or to any subsequent resting sites. On the

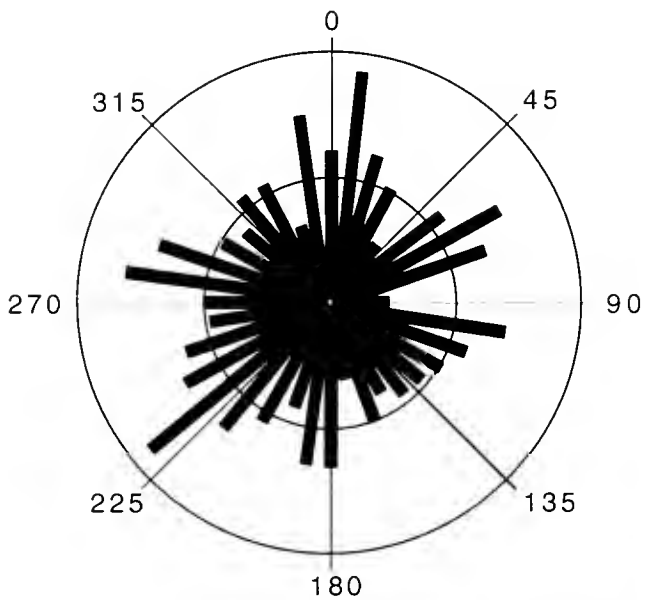

Figure 1. Frequency distribution of directions of all movements made by *Cerithidea muricatus* during all observation periods (n = 366 observations). Radial lines represent 10 and 20 snails, respectively. Directions of movement at each site have been standardized so that the direction of the ocean is zero.

other hand, all snails remained within 1–2 m of the sites from which they were originally released. In fact, 1 y later (24 July 1999), 14 (28%) of our marked snails were found within 3 m of the areas from which they were first collected.

Movement Periodicity

Individuals of *C. muricatus* were equally likely to move during the day as at night during our 24-h observation periods ($\chi^2 < 0.001$; df = 1; $P = 0.99$) (Fig. 2). There were some periods of high snail movement on three dates, and in each case the period of high snail movement occurred during the hours following a rainfall or, in the case of the night of 22–23 August, a period of unusually heavy dew. We observed no correlation between the percentage of snails moving and the level of the tide (Pearson correlation, $r = -0.249$; $t = -1.5426$; $n = 38$; $P > 0.1$) (Fig. 2). There was also no correlation between the percentage of snails moving and the phase of the moon ($r = -0.229$; $t = -1.245$; $n = 30$; $P > 0.2$) (Fig. 3). Again, there were several dates on which a high percentage of snail movement was recorded, and each of these corresponded with a preceding rainfall (Fig. 3).

There was a relationship between movement and rainfall. Overall, 89.1% of snail movements occurred within 12 h following a rainfall. The percentage of snails moving during observation periods within 12 h following rainfall (50.5 ± 23.0%; $n = 15$ periods) was significantly greater than during observation periods that were not preceded by a rainfall within 12 h (1.7 ± 4.11%; $n = 45$ periods; unpaired two-sample t test, $t = 8.12$; df = 14; $P < 0.0001$). To determine whether snail movement decreased with increasing time since rainfall, all observations within 12 h following rainfall for the 24-h observation periods were examined. The percentage of snails moving was greatest shortly following a rainfall and showed a significant linear decrease over time (regression, $y = 76.3 - 4.54x$; $r^2 = 0.35$; $P = 0.044$). Although the percentage of snails that was active decreased following rainfall, there was no change in the rate of movement as the time from rainfall increased (regression, $y = 26.6 - 1.18x$; $r^2 = 0.16$; $P = 0.19$). Snail movement appears to cease more rapidly during daytime than at night; however, our low sample sizes did not permit us to test this statistically.

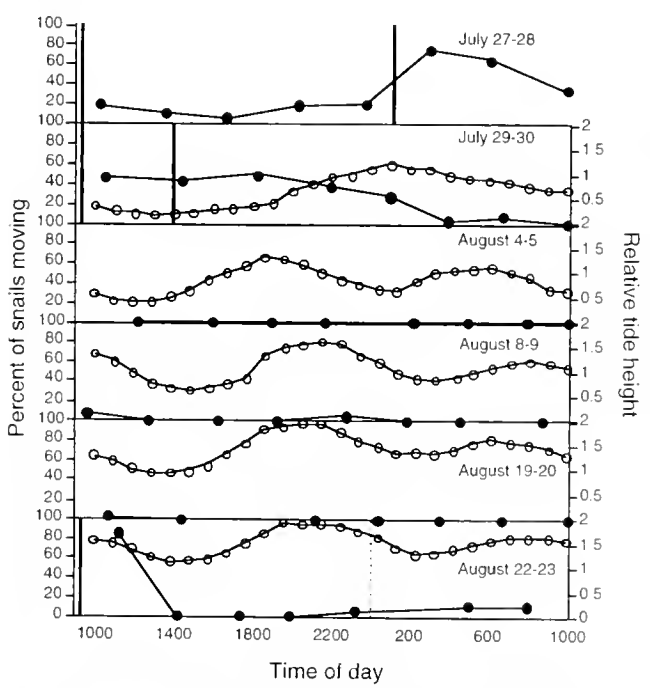

Figure 2. Movement of *Cenchritis muricatus* during the six 24-h observation periods. The percentage of snails moving (solid circles) and relative tide height (open circles) are shown. Tide data from 27–28 July were omitted due to equipment malfunction. Solid vertical lines indicate times of rainfall; dashed vertical line indicates an unusually heavy dew.

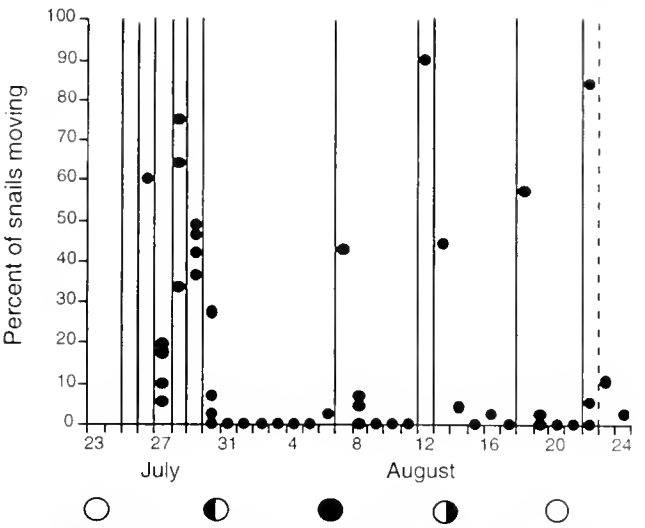

Figure 3. Movement of *Cenchritis muricatus* over the lunar cycle from 26 July to 24 August 1998. For dates on which there were multiple observation periods, the percentage of snails moving during each observation period is indicated. Solid vertical lines indicate dates on which rainfall occurred; dashed vertical line indicates an unusually heavy dew.

Effect of Microhabitat on Location and Movement

When all observations from all snails were pooled, *C. muricatus* exhibited a strong preference for crevices and pits over exposed surface ($\chi^2 = 5324.21$; df = 2; $P < 0.001$). Observations during which snails were found on or under vegetation ($n = 426$) were excluded from this analysis. Of the remaining 2,296 observations, 54.2% were in crevices, 28.0% were in pits, and 17.8% were on the surface, in spite of the fact that only 14.9% of the area consisted of crevices, 8.5% was pitted, and 77.1% was exposed surface. When chi-square tests were performed on microhabitat preferences for each snail independently, 45 of 46 snails showed significant preferences for crevices and pits over exposed surface (all at $P < 0.001$). In 31 cases, crevices was the major contributor to these significant chi-square values, and in 14 cases, pits was the major contributor.

In the absence of rainfall, snails were equally likely to move from all microhabitats (2.5, 1.1, 0.96, and 1.4% from exposed surface, pits, crevices and vegetation, respectively; $\chi^2 = 2.74$; df = 3; $P > 0.1$). Following a rainfall, snails were more likely to move from a resting position on the surface (24.3%) than from pits (9.2%), crevices (9.8%), or vegetation (9.9%; $\chi^2 = 60.7$; df = 3; $P < 0.001$).

Wetting Experiment

There was a significant effect of wetting on the percentage of snails moving (one-way ANOVA: $F = 9.88$; df = 3; $P = 0.001$). However, a Tukey's multiple comparisons test indicated that the

type of wetting treatment (250 ml vs. 500 ml fresh water vs. 250 ml sea water) had no effect on the percentage of snails moving. The percentage of snails moving in all treatment quadrats combined was 63.9 ± 23.0%, as compared with 0 in the control quadrats.

DISCUSSION

This study demonstrated that individuals of *C. muricatus* do not exhibit diurnal, tidal, or lunar periodicity in their movement patterns. Movement in *C. muricatus* appears to occur in response to wetting of the substrate by rain or dew. Individuals of *C. muricatus* occurred preferentially in pits and crevices, and this selection for sheltered microhabitats and a propensity to move only under moist conditions suggest that these behaviors may have evolved as means to reduce desiccation potential in this tropical high-shore species.

Lang et al. (1998) observed that *C. muricatus* spends most of its time in repose, and this study supports that observation. When *C. muricatus* did move, it did not show evidence of directional movements, and it did not move long distances. During our study, we were able to locate all but two of our marked snails, and all were found within 2 m of their original locations. One year later, we were able to find 14 (28%) of our marked snails, all of which were within 3 m of their initial locations. This pattern of short, nondirected movements—a random walk, in effect—appears to maintain the distribution of *C. muricatus* along the shore, both over the short and long term, as suggested by Chelazzi et al. (1988). Underwood (1977) also found that three species of intertidal gastropods moved in random directions, at least over a 1- to 3-day period, and Petraitis (1982) observed that *Littorina littorea* moved randomly except following dislodgment, when movement was aimed at returning the individual to its initial zone on the shore.

We did not observe any evidence of diurnal, tidal, or lunar migration in *C. muricatus* during this 4-wk study. Nocturnal foraging has been implicated in littorinids (Voltolina & Sacchi 1990), and Kaplan (1988) suggested that *C. muricatus* might migrate downshore to forage on algae in the intertidal zone at night, when temperatures are cooler and the potential for desiccation is reduced. However, this was not the case in our study. One possible explanation for the absence of nocturnal migrations in *C. muricatus* may be that its food source, which most likely consists of endolithic and/or epilithic algae (Norton et al. 1990, McQuaid 1996), is adequate in the supralittoral fringe.

The absence of tidal rhythmicity to movement in *C. muricatus* is not unexpected. The supralittoral fringe zone occupied by *C. muricatus* is highest on the shore and is never inundated by tides except during severe storms, none of which occurred during our study. Therefore, *C. muricatus* does not receive an exogenous cue from the tide itself, which might initiate movement, and there does not appear to be an endogenous cue for tidal periodicity in movement behavior. Zann (1973) also noted the absence of tidal periodicity to movement in supralittoral gastropods that were not subjected to regular tidal action.

Although clearly not ubiquitous, patterns of diurnal or tidal periodicity of movement are known from littorinids. Ohgaki (1989) noted that *Nodilittorina exigua* in Japan exhibits upward migrations with the high tide, and foraging activity in *L. littorea* is also associated with the tide (Newell et al. 1971). *L. scutulata* exhibits diurnal periodicity in foraging activity (Voltolina & Sacchi 1990). On the other hand, Little (1989) reviews foraging activity in a variety of littoral molluscs and lists only three species that exhibit foraging activity with no regular pattern relating to tides or time of day; all three of these species are littorinids.

Unlike many earlier studies on movement in intertidal molluscs, our observations on *C. muricatus* spanned an entire lunar cycle. Nevertheless, we did not see any evidence of movement periodicity associated with the lunar cycle during our study. In some species of littorinids, reproductive behavior has been linked to lunar cues (Hughes & Roberts 1980, Berry 1986). In at least one species of high-shore littorinid, there is evidence of a seasonal downshore migration associated with spawning (Ohgaki 1988). *C. muricatus* may display a similar seasonal reproductive migration, which would not necessarily have been observed during our study. In fact, in June–July 1998 and 1999, large aggregations of *C. muricatus* were observed low on the shore at the water's edge, well below this species' typical supralittoral fringe habitat (unpublished data). Upshore migrations were subsequently observed in mid-July. It is suspected that this migration is associated with reproduction; however, this has not yet been confirmed.

There was no evidence of homing to specific sites on the rock in *C. muricatus*. However, the fact that snails were found at or near their initial locations a year later, following a massive downshore migration, suggests that there may be a mechanism by which individuals are able to return to the same general location on the shore. Other littoral molluscs that migrate vertically with the tide are able to return to the same approximate locations following downshore foraging movements, and this is probably regulated by a complex of stimuli (Underwood 1979, Chelazzi et al. 1988).

Movement in *C. muricatus*, as in several other species of supralittoral gastropods, appears to occur exclusively when the rock is wet. The results of the wetting experiment indicate that the volume and salinity of the water was unimportant in eliciting movement behavior. Because *C. muricatus* occupies the highest zone on the rocky shore in Jamaica, this area is rarely reached by wave splash and is never inundated by tides. On the other hand, rainfall and dew can wet this zone on a reasonably regular, if unpredictable, basis. If rainfall occurs in midday, the rock can dry quite rapidly, but at night the rock may remain damp for several hours. Although we did not measure relative humidity, *C. muricatus* may also move when relative humidity of the air is very high (R. McMahon, pers. comm.).

Individuals of *C. muricatus* appear to take advantage of the wet rock for their foraging excursions. Foraging when the rock is wet may be an adaptive mechanism to reduce desiccation potential, which is normally severe in this zone. In addition, rainfall may facilitate foraging by softening the epilithic biofilm, the probable food source of *C. muricatus*. Chapman (1994a) reported that *Nodilittorina pyramidalis*, a high-shore species from Australia, also feeds opportunistically when the rock is wet, either by rain or tide, and becomes inactive when the rock dries. The limpet *Siphonaria pectinata* forages when the substrate is very damp or wet, and when the relative humidity drops below 75% during foraging, the limpet stops moving and attaches to the rock even if it has not returned to its home scar (Ocana & Emson 1999). This is presumably an adaptive mechanism for limiting water loss until the limpet can return to its home scar (Ocana & Emson 1999).

Individuals of *C. muricatus* preferentially occupied pits and crevices as opposed to exposed surfaces on the rock. Gastropods occupying the supralittoral fringe zone on tropical shores are exposed to direct sunlight and extreme temperatures during their prolonged periods of emersion. The preferential use of pits and

crevices by *C. muricatus* may be an adaptation to mediate extreme environmental conditions. Individuals of *C. muricatus* occupying crevices tend to have lower ambient temperatures than those on exposed rock surfaces (Lang et al. 1998). Crevices and pits may also provide some degree of shade in a habitat otherwise fully exposed to direct sunlight, and snails occurring in these microhabitats may be able to trap more moisture. Snails found on or under vegetation may also experience reduced desiccation potential as a result of shading and reduced contact with the hot rock, although they may also be farther from their food source.

Preferential use of certain microhabitats, particularly crevices, has been reported in several other littorinids (Garrity 1984, Underwood & Chapman 1992), although explanations for such preferences vary. Our data are consistent with other studies of tropical littorinids, which found that pits and crevices provided protection from heat stress and desiccation (Garrity 1984, Peckol et al. 1989). To the contrary, studies of temperate littorinids suggest that the use of pits and crevices do not protect against heating or desiccation (Chapman 1994b) but may provide a more abundant food source (Voltolina & Sacchi 1990) or protection from wave action (Raffaelli & Hughes 1978). Except during severe storms, *C. muricatus* at our study site in Jamaica is not at risk from wave action, but desiccation potential on this tropical shoreline is high and may pose a serious risk to individuals of this species (Lang et al. 1998). Littorinids occurring in crevices may also be protected from predation pressure (Catesby & McKillup 1998); however, predation on *C. muricatus* is probably a relatively minor selection pressure at our study site.

Individuals of *C. muricatus* on exposed rock surfaces were more likely to move when the substrate was wet, whereas individuals in more sheltered microhabitats (pits, crevices, or vegetation) were more likely to remain stationary. This difference in behavior as a function of microhabitat may reflect the more extreme desiccation potential on exposed rock surfaces, and moving from an exposed surface to a sheltered habitat may be advantageous. Alternatively, snails on exposed surfaces may be more energetically stressed due to increased heat and desiccation potential and may be more inclined to resume foraging when the rock is wet than are snails in more sheltered microhabitats. When the rock was dry, very few snails moved, regardless of their resting microhabitat.

Foraging activity in *C. muricatus* appears to be an opportunistic strategy in which snails are able to take advantage of favorable conditions during which desiccation potential is reduced. For molluscs found in the supralittoral fringe on tropical shorelines, desiccation potential may be an important selective pressure, although other abiotic factors may be equally or more important because most highshore gastropods are able to tolerate greater levels of desiccation than they ever experience in their natural habitats (R. McMahon, pers. comm.). Substratum temperatures of the exposed rock surface in the supralittoral fringe at our study site in July reached a high of 45°C (Minton & Gochfeld in press), whereas the heat coma temperature of *C. muricatus* is 42.1°C (Britton 1992). Therefore, *C. muricatus* is exposed to potentially lethal temperatures for extended periods of time and has evolved behavioral mechanisms that minimize its potential for desiccation and heat stress, including preferential use of sheltered microhabitats and movement only when the rock surface is damp or wet.

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A MOLECULAR ASSAY IDENTIFIES MORPHOLOGICAL CHARACTERS USEFUL FOR DISTINGUISHING THE SIBLING SPECIES *LITTORINA SCUTULATA* AND *L. PLENA*

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ABSTRACT Sibling species *Littorina scutulata* and *L. plena* are difficult to distinguish in the field. Here we present a new molecular tool and use it to evaluate the discrete and quantitative morphological characters that have been proposed as diagnostic. We collected 385 snails of both species from 11 sites in Washington state and used restriction enzyme digestion of a PCR-amplified, 480 bp fragment of the mitochondrial cytochrome b gene to distinguish the species. This new molecular assay produces species-specific restriction fragment patterns that correspond with identification of males by penis morphology. To evaluate the usefulness of morphological characters, we scored three discrete shell characters (presence of basal band, presence of basal ridge, and size of checker pattern) as well as tentacle coloration. The four discrete characters differ significantly between the two species, though none is completely diagnostic. Tentacle coloration is the most reliable character and may be combined with the shell characters for successful identification. The two species also differ significantly in overall size and in three out of five size-independent shell shape measurements, with *L. scutulata* having larger, taller-spired shells with narrower apertures. However, shell shape does not separate the species well because of intraspecific variation, and it is unlikely to be useful for species identification. Further analysis suggests that at least some of this intraspecific variation is genetic rather than environmental. The distributions of the two species overlap broadly in Washington, though only *L. plena* was found in exposed outer coast habitats, contrary to previous work.

KEY WORDS: *Littorina scutulata*, *L. plena*, species identification, sibling species, molecular systematics

INTRODUCTION

The taxonomic history of the *Littorina scutulata* species complex, a group of sympatric intertidal prosobranch gastropods in the Northeastern Pacific, has been complicated by morphological similarity across species and variation within species. Currently two sibling species are recognized, *L. scutulata* (*sensu stricto*) Gould 1849 and *L. plena* Gould 1849, which are distinguished on the basis of reproductive characters, including penis, pallial oviduct, and egg capsule morphology (Murray 1979, Mastro et al. 1982, Reid 1996). These characters, however, are difficult to use for the non-destructive field identification that is necessary for many ecological studies. Reproductive characters cannot be used for juveniles, and we have found the dissection necessary for examining pallial oviduct morphology to be difficult, especially in small specimens.

Other diagnostic morphological characters have been proposed. Three discrete shell characters have been described: a pale basal band (Murray 1982, Reid 1996, Chow 1987, Rugh 1997) and a narrow basal ridge (Rugh 1997), both found more often in *L. plena*, and the pattern of checkers on many shells, which tend to be smaller in *L. plena* than in *L. scutulata* (Reid 1996, Rugh 1997). Rugh (1997) was able to use these three shell characters alone to correctly identify 17 male specimens of both species from southern California. Reid (1996) described differences in tentacle coloration: *L. scutulata* individuals tend to have "transverse black bands and flecks," while *L. plena* tend to have a "broad, unbroken black stripe with transverse flecks, or all black." Murray (1982) described a set of discriminant functions of four quantitative shell measurements that correctly classified 96% of specimens. Further principal component analysis by Murray (1982) showed *L. scutulata* shells to be generally taller with narrower spire angles and shorter aperture openings relative to shell height. Chow (1987) combined three quantitative shell measurements with number of whorls, presence of a basal band, and presence of tesselation in another discriminant function analysis. This analysis correctly classified 92% of specimens, but only when using snails from one habitat; combining specimens from different habitats introduced too much intraspecific variation to allow correct classification. Chow (1987) also found *L. scutulata* shells to be larger, narrower, and less likely to have a basal band, which agreed with past work. Other characters used with varying success to distinguish these species include spiral sculpture on the shell and radular characters (Reid 1996, Mastro et al. 1982).

Mastro et al. (1982) found eight polymorphic allozyme loci at which the two species differ in their allele frequencies. However, none of these loci was diagnostic. No other molecular studies to date have identified a reliable molecular character at a polymorphic locus that distinguishes the two species.

The previous studies of morphological differences used specimens that were positively identified using reproductive characters, thus excluding pre-reproductive animals. Here we present a molecular technique for identifying individuals of all ages using mitochondrial DNA. We use this tool to evaluate the reliability of characters that can be observed on intact animals: the three discrete shell characters described above, tentacle coloration, and quantitative shell shape differences.

MATERIALS AND METHODS

We collected 385 snails of both species from 11 areas around Puget Sound and the outer coast of Washington state in January 1998 (see Fig. 1) and kept them alive until DNA extraction. Animals of all sizes, including juveniles, from within a randomly chosen, small (~1 m^2) area of rocky shore habitat were collected. Individuals anesthetized in 7% $MgCl_2$ seawater solution were

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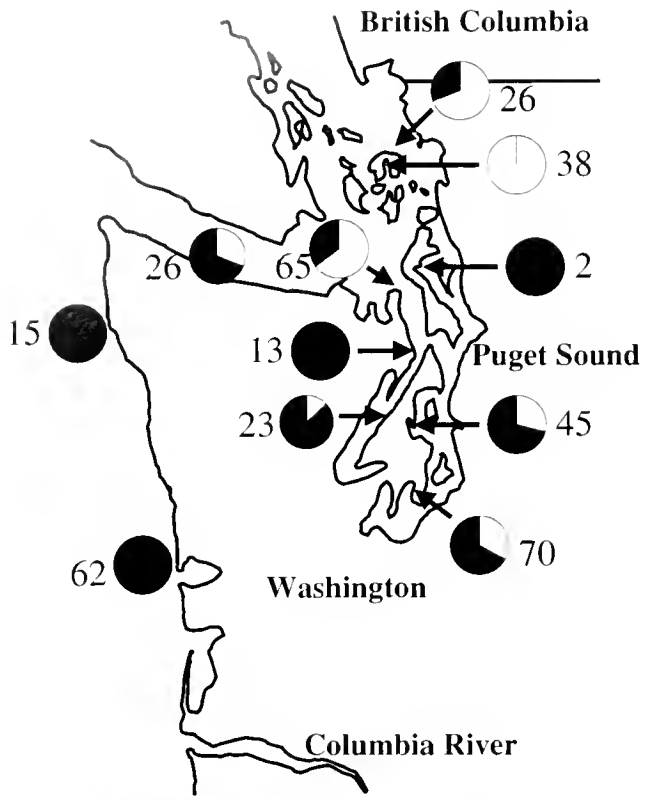

Figure 1. Map of western Washington state showing collection sites in Puget Sound and on the outer coast. Pie diagrams show relative abundance of *L. plena* (dark) and *L. scutulata* (light) with the total sample size for each site.

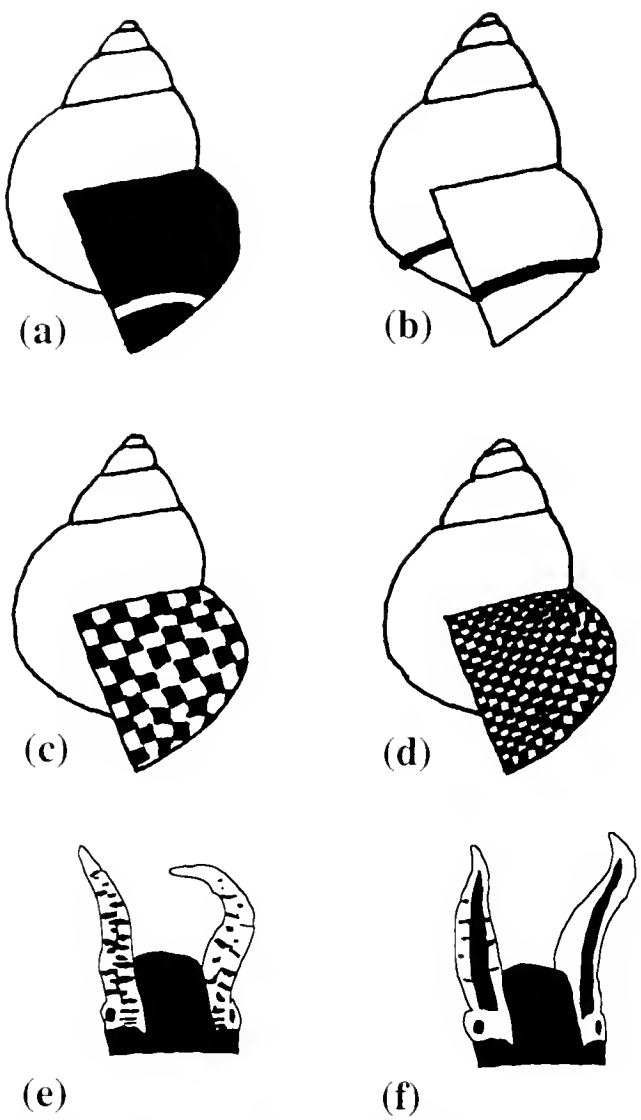

Figure 2. Discrete characters. (a) A pale basal band and (b) basal ridge are found more often in *L. plena*. (c) A pattern of large checkers typifies *L. scutulata*, while (d) small checkers are found more often in *L. plena*. (e) Two tentacle coloration patterns found in *L. scutulata*: transverse bands (left) and bands and spots (right). (f) Two tentacle patterns found in *L. plena*: a broad central stripe with bands (left) and without bands (right).

scored for three discrete shell characters (Fig. 2a–d): basal band present or absent, basal ridge present or absent, and checkers large, small, or absent. Large checkers lie in five to 12 spiral rows, depending on shell size, while small checkers number more than 10 spiral rows. Tentacle coloration was scored in one of seven categories: dark transverse bands, spots and bands, spots, dark central stripe, central stripe with bands, no color, or all dark (Fig. 2e–f; see also Reid 1996). In all further analyses, the first three categories were grouped as "transverse bands," and the last four were grouped as "central stripe." Individuals' sex was also recorded and males scored as *L. plena* or *L. scutulata* penis type. *L. scutulata* penes are relatively short with a terminal bifurcation, while *L. plena* penes are longer, often coiled, with a bifurcation near the base (Reid 1996).

Animals were then sacrificed for molecular analysis. Using the extraction protocol and PCR primers described in Kyle and Boulding (1998), a 480 bp fragment of the mitochondrial cytochrome-b gene was amplified for each individual. These were then digested for two hours using the restriction enzyme *Alu* I and the digests run on a 2% agarose gel. To predict restriction sites for the two species, we examined 36 *L. plena* and 18 *L. scutulata* haplotype sequences from Kyle and Boulding (2000) (Genbank accession nos. AF077238–AF077291). Identification of these sequences by Kyle and Boulding (2000), however, depended on a single *L. plena* sequence from Reid et al. (1996) (Genbank accession no. U46815), so our analysis also functioned to confirm the identification of those sequences. We expected fragments of 161, 233, and 86 bp for *L. scutulata* and 109, 15, and 356 bp for *L. plena*.

Eight quantitative shell measurements were taken from each individual using a dissecting microscope connected to computer imaging software (Fig. 3). Since six of these measurements are lengths, they were combined into three non-dimensional ratios to remove the effect of overall shell size as follows: Relative aperture height = Aperture height / Shell height; Whorl ratio = Whorl n–2 / Whorl n–1; and Aperture shape = Short axis / Long axis. Statistical tests and discriminant function analysis used these three ratios as well as spire angle and aperture angle (Fig. 3).

In an attempt to improve differentiation of the two species based on shell morphology, further discriminant function analyses were done using different sets of variables. In the first, size was included specifically by adding shell height to the three ratios and two angles, providing six variables for discriminant analysis. In the second revision, the original six linear measurements were not combined into ratios as above, but rather normalized by the

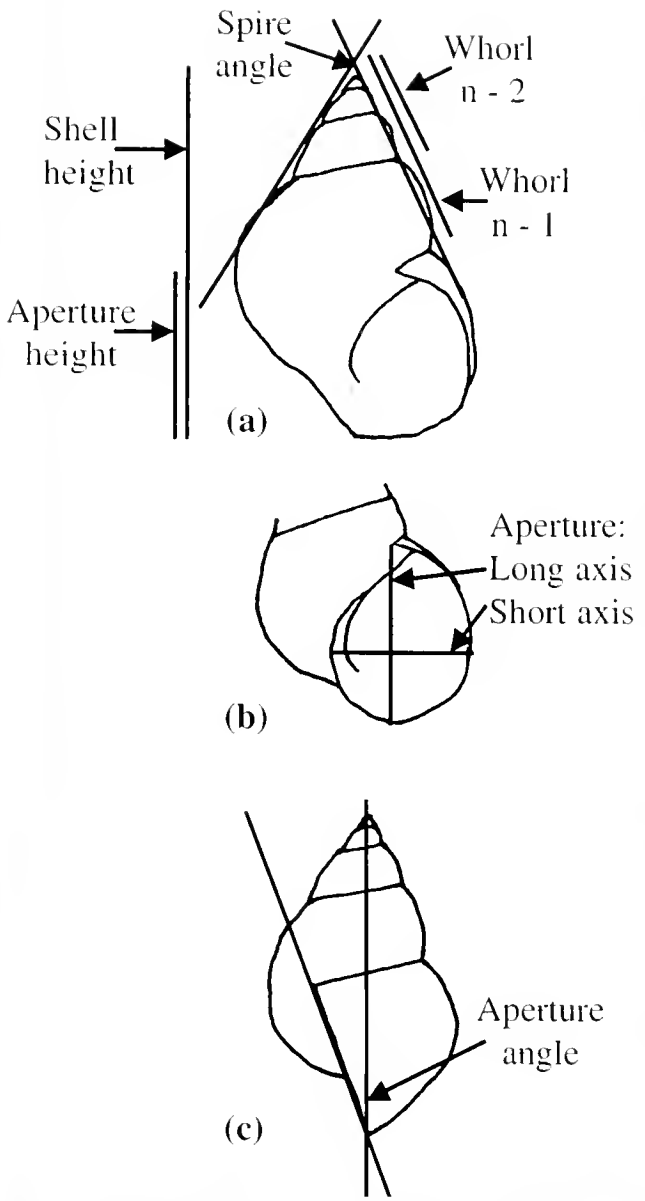

Figure 3. Quantitative shell measurements. Shells were viewed through a dissecting scope and video camera connected to a computer and measurements were taken using imaging software. The two angles were measured in degrees and the six lengths in mm.

method of Clarke et al. (1999). The effects of size were removed from each linear measurement by dividing the geometric mean of each specimen's measurements:

$$\text{geometric mean} = 10\text{^}[(\log_{10}(x_1) + \log_{10}(x_2) + \ldots + \log_{10}(x_6))/6]$$

where x_1 through x_6 are the original linear measurements. This provided two angles and six normalized linear measurements for discriminant analysis.

The third attempt to improve the discrimination used only the specimens from six sites at which both species were found (see Fig. 1) and analyzed the three ratios and two angles as above. Finally, another analysis was performed on data limited to three sites of similar habitat following Chow (1987). These three sites are the three southern Puget Sound sites shown in Figure 1. All are protected shores at which both species were found.

RESULTS

Restriction enzyme digestion with *Alu* I produced two discrete fragment length patterns as expected: one pattern with three closely spaced bands (*L. scutulata*) and another with two widely spaced bands (*L. plena*; the 15 bp fragments typically migrated off the gel). These corresponded precisely with identification of males by penis morphology (n = 48 *L. scutulata* and 71 *L. plena*). This supports the identification of sequences in Kyle and Boulding (2000) and suggests that these restriction sites are consistent across haplotypes within each species. The following analyses are based on these 119 males as well as females identified using only restriction digest and males identified by penis morphology alone. Sample sizes vary because of damage to some shells during DNA extraction.

The frequency of each of the four discrete characters differs significantly between the species (Table 1) consistent with Rugh (1997) and Reid (1996). The differences remain significant following Bonferroni correction of the original p-values from four separate contingency table analyses (Rice 1989). However, no single character completely separates the two species. The shell characters were often not visible because of shell erosion from wave action or fungal or other epiphytic growth, creating a potential bias toward identification as *L. scutulata* from the first two characters. Many undamaged shells also lack any checker pattern. Tentacle coloration, because it was always scorable in live animals, was the most reliable discrete character.

Following Bonferroni correction of the original p-values from six separate two-tailed *t*-tests (Rice 1989), the species differ significantly in four shell measures (Table 2). *L. scutulata* shells are significantly larger than *L. plena* shells. Of the five size-independent shell measures, the two species differed significantly in spire angle, whorl ratio, and aperture shape. These results are consistent with those of Murray (1982) and Chow (1987), confirming that *L. scutulata* shells are larger, narrower, and taller-spired with narrower apertures. Relative aperture height, though not significantly different, also follows the trend found by Murray (1982). Three of these shell measures, shell height, whorl ratio, and aperture shape, remain significant when considering only the six sites at which both species were found (Table 2). The difference is lost for spire angle. However, the difference in relative aperture height reverses and becomes statistically significant when only these sites are considered.

Combining specimens from all the collection sites, these five

TABLE 1.

Species differences for four discrete characters. Species were identified by restriction enzyme digest and penis morphology. Data given are number of specimens (percentage) in each category. The p-values are Bonferroni corrections (Rice 1989) of separate χ^2-tests.

Character	State	*L. scutulata*	*L. plena*	p-value
Basal band	present	18 (12.4)	124 (51.7)	<0.001
	absent	127 (87.6)	116 (48.3)	
Basal ridge	present	23 (15.9)	149 (62.1)	<0.001
	absent	122 (84.1)	91 (37.9)	
Checker pattern	large	122 (84.1)	25 (10.4)	<0.001
	small	5 (12.4)	93 (38.8)	
	absent	18 (12.4)	122 (50.8)	
Tentacle color	transverse bands	130 (89.7)	10 (4.2)	<0.001
	central stripe	15 (10.3)	230 (95.8)	

TABLE 2.

Quantitative shell measurements. Numbers given are mean (standard deviation), and the p-values are Bonferroni corrections (Rice 1989) of separate two-tailed *t*-tests. For each measure, the first row includes specimens from all sites, and the second row includes only specimens from the 6 sites at which both species were found.

Measurement all sites: 6 sites:	*L. scutulata* n = 142 n = 104	*L. plena* n = 210 n = 146	p-value
Shell height (mm)	8.1 (1.6)	6.3 (1.3)	<0.001
6 sites	7.4 (1.2)	6.3 (1.2)	<0.001
Spire angle (deg.)	54.2 (4.0)	55.5 (5.8)	0.045
6 sites	54.4 (4.1)	54.5 (5.5)	>0.5
Aperture angle (deg.)	24.0 (2.4)	23.8 (2.1)	>0.5
6 sites	23.9 (2.4)	23.9 (2.2)	>0.5
Relative ap. Height	0.516 (0.038)	0.519 (0.039)	>0.5
6 sites	0.528 (0.034)	0.513 (0.039)	0.01
Whorl ratio	0.595 (0.163)	0.555 (0.045)	<0.001
6 sites	0.607 (0.188)	0.568 (0.002)	0.045
Aperture shape	0.703 (0.038)	0.726 (0.037)	<0.001
6 sites	0.697 (0.040)	0.721 (0.037)	<0.001

shell measures were used in a discriminant function analysis without much success: the function correctly classified only 69% of *L. scutulata* and 66% of *L. plena* specimens. This seems to be the result of overlapping intraspecific variation for all of the characters.

Attempts to improve the discriminant function analysis were marginally successful. Because *L. scutulata* shells were significantly larger, including shell height improved posterior classification to 76% for *L. scutulata* and 81% for *L. plena*. Normalizing the linear measurements by the geometric mean (Clarke et al. 1999) improved classification of *L. scutulata* to 82%, but reduced successful classification of *L. plena* to 62%. Limiting the analysis to the six sites where both species were found made no improvement over the original dataset: 69% for *L. scutulata* and 68% for *L. plena*. Finally, restricting the analysis to the three protected Puget Sound sites only slightly improved classification: 66% for *L. scutulata* and 74% for *L. plena*. These results are summarized in Table 3.

The geographic ranges of these two species overlap significantly in Washington, although *L. scutulata* is found only in moderately exposed to sheltered areas of Puget Sound while *L. plena* is found from sheltered sites in Puget Sound to the exposed headlands of the outer coast (Fig. 1). This conflicts with Reid (1996), who found only *L. scutulata* in exposed habitats.

TABLE 3.

Varying success of discriminant function analyses of quantitative shell measurements. Correct posterior classification percentages are given along with total sample size of specimens for each species.

Variable combination	L. scutulata	L. plena
2 angles, 3 ratios	69 (n = 142)	66 (n = 210)
2 angles, 3 ratios, shell height	76 (n = 142)	81 (n = 210)
2 angles, 6 linear measurements normalized by geometric mean	82 (n = 142)	62 (n = 210)
2 angles, 3 ratios from sites with both species	69 (n = 104)	68 (n = 146)
2 angles, 3 ratios from protected habitats only	66 (n = 38)	74 (n = 99)

DISCUSSION

L. plena individuals tend to have a pale basal band, basal ridge, and small checker pattern on their shells and a broad central stripe on their tentacles, while *L. scutulata* individuals lack the basal band and ridge, have a larger checker pattern, and have transverse bands and spots on their tentacles (see Fig. 2). In addition, *L. scutulata* shells are larger and taller-spired with narrower apertures. However, we do not believe the discrete shell characters alone to be sufficient, as did Rugh (1997), nor do we believe shell shape differences to be diagnostic, as did Murray (1982). Though the species differ significantly in these characters, intraspecific variation and shell damage may confound identification.

No combination of quantitative shell measurements in a discriminant function analysis provided reliable identification. The most successful analysis used shell size explicitly, which could potentially bias the identification of different ages of snails, so its utility in ecological studies would be limited. Neither method of removing the effects of size matched the discrimination ability of Murray (1982) or Chow (1987), suggesting that interspecific differences are truly confounded by intraspecific variation. One attempt to eliminate some intraspecific variation by examining only sites at which both species were found did not improve the results, so this dataset does not show any evidence for character displacement in shell shape. Limiting the analysis to a single habitat type was not successful either. Since this analysis attempted to reduce environmental phenotypic variation, the result suggests that at least some of the intraspecific variation observed is genetically based.

One likely explanation for our different results is that these previous studies used only reproductive, hence larger, animals. Species differences may become more apparent as the snails grow (Reid 1996; Hohenlohe pers. obs.), and ecological applications typically require identification of animals of all ages. Murray (1982), Chow (1987), and Rugh (1997) primarily used specimens from California, and geographic differences may also play a role. Chow (1987) also combined discrete and quantitative characters in a single analysis, which was not done here.

We found *L. plena* occupying a wider range of habitats in Washington, from sheltered Puget Sound sites to the exposed outer coast. In contrast, *L. scutulata* was found only on sheltered to moderately exposed shores. This result conflicts with some previous work (Reid 1996) but is consistent with other data on species distributions (Hohenlohe 2000). This discrepancy is investigated further in Hohenlohe (2000).

These species can be distinguished non-destructively by combining the characters discussed here. Male penis morphology can be easily examined by holding the snail upside down, underwater, under a dissecting microscope. For females and non-reproductive males, tentacle coloration is the most reliable character and can be combined with shell characters on undamaged specimens. For positive identification of all ages and both sexes, restriction enzyme digestion of cytochrome b with *Alu* I is straightforward and reliable and provides a diagnostic character independent of morphology.

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ACUTE THERMAL TOLERANCE IN INTERTIDAL GASTROPODS RELATIVE TO LATITUDE, SUPERFAMILY, ZONATION AND HABITAT WITH SPECIAL EMPHASIS ON THE LITTORINOIDEA

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ABSTRACT Acute thermal tolerance was determined as mean heat coma temperature (HCT) for samples of 30 to 36 individuals each (range = 17–71) of 60 species of intertidal snails from rocky shores (40 species), mangroves (17 species) and salt marshes (3 species) at 10 collecting sites across a latitudinal range of 12.5°– 54.6° (54.61° N – 35.00° S). HCT was quadratically correlated to latitude, increasing with decreasing latitude above 25°, but remaining constant in tropical latitudes below 25°, indicating that thermal tolerance of tropical species is not as latitudinally influenced as in subtropical and temperate species, or, alternately, that tropical species have attained physiologically near maximal levels of thermal tolerance. Mean HCT was greater in the Littorinoidea than in the other six superfamilies tested, perhaps accounting its dominance of high-shore habitats where increased thermal tolerance reduces water loss due to evaporative cooling. Similarly, the high-shore superfamilies, Cerithoidea and Neritoidea, also had high mean HCT values. Increasing thermal selection with shore height was reflected in increasing HCT among low-, mid- and high-shore species. HCT did not differ among littorinoidean and nonlittorinoidean snails on low- or high-shores, but was elevated in mid-shore littorinoideans, suggesting retention of the elevated thermal tolerance of this latter group's high-shore ancestors. In contrast, progressive HCT increase with shore height among mid- and high-shore nonlittorinoideans suggests that they evolved from low-shore ancestors. Among all taxa, thermal tolerance increased progressively across rocky shores, mangroves and salt marshes. Littorinoidean snails had greater HCT than nonlittorinoidean snails among all three habitats, suggesting that mangrove and salt-marsh littorinoideans retain the elevated thermal tolerance of their high rocky shore ancestors. In contrast, HCT of nonlittorinoidean snails increased across rocky shore, mangrove and salt marsh habitats suggesting that they pose different thermal selection pressures on species evolving into them from the low-shore.

KEY WORDS: Gastropoda, heat coma temperature, intertidal gastropods, Littorinoidea, thermal adaptation, thermal tolerance, zonation

INTRODUCTION

A plethora of studies describe the thermal tolerance of intertidal invertebrates relative to vertical zonation. Sessile species, such as barnacles, present a fairly clear pattern of increased thermal tolerance with increasing zonation level, elevated thermal tolerance of higher zoned species considered an adaptation to the elevated ambient temperatures associated with insolation during prolonged tidal emersion (reviewed by Newell 1979). The situation for mobile intertidal gastropods is more problematic because they seek temperature microrefugia (McMahon & Yipp 1992, Chapman & Underwood 1996) and are active only during periods of reduced thermal stress (Ruwa & Jaccarini 1988). Thus, their acute upper thermal tolerance limits may not consistently correlate with their vertical distributions (for a review see McMahon 1990). Further, intertidal gastropod mobility results in species having broad, sympatric vertical distributions with extensive species overlap (Broekhuysen 1940, McMahon 1990, Lang et al. 1998) and species vertical distributions changing with season (Vaughn & Fisher 1992). Thus, assignment of gastropods to narrowly defined vertical zones is difficult at best and often impossible.

In a review of their resistance and capacity adaptations, McMahon (1990) hypothesized that intertidal gastropods can be divided into three major zonational groups characterized by unique suites of physiological adaptions. Sublittoral to lower eulittoral species (referred to hereafter as low-shore species) are primarily aquatic, generally not ranging above the mean low tide mark, where they are submerged most of the time (>86%). Thus, low-shore species appear to be adapted to an aquatic existence and usually unable to sustain aerial gas exchange when emerged. Their low shore habitat is more temperature-stable than periodically emersed eulittoral and eulittoral fringe-maritime zone habitats (McMahon 1988, 1990).

Eulittoral gastropod species (referred to hereafter as mid-shore species) are restricted to areas between high and low tide marks where they experience only short-term tidal emersion (<12 h), spending 14–86% of the time in air depending on shore height. Thus, mid-shore species lead an amphibious existence, their adaptations hypothesized to center on remaining active during emersion and immersion. Thus, their aerial and aquatic O_2 uptake rates are generally equivalent even though aerial gas exchange results in extensive evaporative water loss (McMahon, 1990). When emersed in direct sunlight, mid-shore gastropods may evaporatively cool to prevent lethal tissue warming, rapidly rehydrating during subsequent tidal inundation (McMahon 1990, McMahon & Yipp 1992, Lang et al. 1998).

Upper eulittoral fringe-maritime zone species (referred to hereafter as high-shore species) are restricted to regions above the high tide mark where they experience prolonged emersion (8–10 days) between successive spring tides (emersed for 86–98% of the time). Adaptations in this group are hypothesized to center on tolerance of prolonged emersion including elevated temperature tolerance and reduction in O_2 uptake rates during emersion which conserve organic energy stores and minimize evaporative water loss. Thus, some studies indicate that high-shore gastropods have higher acute upper thermal limits than mid-shore species (Broekhuysen 1940, Cleland & McMahon 1990, McMahon 1990, Britton 1992), hy-

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pothesized to allow avoidance of water loss due to evaporative cooling during prolonged emergence (McMahon 1990).

There is little sympatry among gastropod species occupying low-, mid- and high-shore habitats, but much sympatry within them (McMahon 1990), suggesting that adaptation to the physical conditions of any one zone restricts species from occupying others. Thus, it is hypothesized that low temperature tolerance in low-shore species prevents them from surviving the elevated temperatures experienced on mid- or high shores during isolation. Conversely, modification of the respiratory system for aerial as well as aquatic gas exchange is hypothesized to be a liability to mid- and high-shore species in the almost purely aquatic low-shore habitat. Mid-shore snails greatly increase foraging time by remaining active when tidally emersed and appear to have evolved mechanisms, including evaporative cooling and large body size, that allow regulation of body temperature at metabolically efficient levels during insolation. While their acute upper thermal limits are generally greater than those of low-shore species, mid-shore gastropods' capacity for evaporative cooling is hypothesized to have prevented their evolution of the extremely elevated temperature tolerances characteristic of high-shore species (McMahon 1990).

All of the above hypotheses regarding physiological correlates with species zonation patterns hinge on the assumption that thermal tolerance increases with height of occupation on the shore. This assumption is based on a series of studies on the relationship between vertical zonation and thermal tolerance in intertidal gastropods limited to a single shore. On a single shore, the number of species available for comparison is limited, being maximally 6 to 14 species (Broekhuysen 1940, Evans 1948, Fraenkel 1966, 1968, McMahon & Britton 1985, Cleland & McMahon 1990, McMahon 1990, Britton, 1992). Low species numbers on any one shore leads to difficulties in data interpretation as anomalously high or low thermal tolerance values for one or two species make zonational thermal tolerance patterns difficult to discern (for examples see Broekhuysen, 1940, Evans 1948, Fraenkel 1966, 1968, McMahon & Britton 1985, Britton 1992, reviewed by McMahon 1990).

Analysis of data for a restricted number of species from a single or just a few shores also prevents elucidation of latitudinal influences on thermal tolerance and empirical comparisons among taxonomic groups. Only two investigations of intertidal gastropod thermal tolerance have involved two or more geographically separated shores. Fraenkel (1968) studied gastropod thermal tolerance on shores in Bimini, Bahamas, Ocean Springs, Mississippi, and Woods Hole, Massachusetts and Stirling (1982), on shores in Hong Kong and Dar es Salaam, Tanzania. In both studies, differences in the thermal tolerance of the gastropod fauna were recorded between shores, but too few shores were investigated to reveal latitudinal trends. Further, cross-comparison of gastropod thermal tolerance data from studies by different authors on geographically separate shores is also problematic because of incompatible methodologies (Fraenkel 1968, Stirling 1982).

In order to empirically assess the effects of zonation level, latitude, habitat, and taxonomic position on the thermal tolerance of intertidal snails, I investigated acute upper thermal limits in 60 gastropod species from ten geographically separate shores over a latitude range of 12.5 – 54.61°, encompassing three different intertidal habitats (i.e., rocky shore, mangrove and salt marsh) and seven different superfamilies. These data were statistically evaluated for differences in upper thermal limit among tested species relative to latitude, and, with latitude as a covariant, among zonation levels, habitat types and taxa on the superfamilial level.

MATERIAL AND METHODS

Species Collection

Thermal tolerance determinations were carried out on samples of 60 intertidal gastropod species (Table 1) collected at 10 geographically separate sites ranging from 54.61° N to 35.00° S latitude including: Robin Hoods Bay, England (54.61°N, number of species (n) = 6); Filey Head, England (54.21°N, n = 2); Porth Towyn, Wales (53.04°C, n = 1); Woods Hole, Massachusetts (41.54°N, n = 1); Gulf Shores, Alabama (30.35°N, n = 1); Port Aransas, Texas (28.84°N, n = 2); Hong Kong (22.37°N, n = 16); Jamaica (18.46°N, n = 13); Darwin, Australia (12.50°S, n = 12); and Albany, Australia (35.00°S, n = 6). Forty species were from rocky shores, 17 from mangroves and three from salt marshes. Of rocky shore species, 18 occurred on the high shore, 13 on the mid-shore and nine on the low shore. Of 17 tested mangrove species, six were high-shore, nine were mid-shore and two were low-shore species. Of three tested salt marsh species, one was a high-shore and two were low-shore species (Table 1). The tested species included seven superfamilies: 25 species of Littorinoidea, 13 species of Cerithoidea, nine species of Neritoidea; four species of Trochacea; four species of Muricoidea; four species of Ellobioidea; and one species of Onchidoidea (Table 1).

Determination of Heat Coma Temperature

Upper thermal tolerance limit was determined as "heat coma temperature" (HCT) using a method (Cleland & McMahon 1990, McMahon & Britton 1985, McMahon & Russell-Hunter 1981, McMahon 1990) modified from that previously used for intertidal gastropods (Gowanloch & Hayes 1926, Broekhuysen 1940, Evans 1948, Southward 1958). At heat coma temperature, gastropods lose normal nervous function leading to muscle paralysis manifested by cessation of locomotion, loss of substratum attachment, ventral-medial lateral curling of the foot; and cessation of movement including tentacles and radular activity (Cleland & McMahon 1990, McMahon 1976, 1990, McMahon & Payne 1980, McMahon & Britton 1985, McMahon & Cleland 1990, McMahon & Russell-Hunter 1981).

Adult specimens of each species for which HCT was determined were collected at low tide and returned immediately to the laboratory. A randomly chosen subsample was randomly divided into five to six subsamples of five to six individuals each which were placed into separate, 50 ml test tubes with 40 ml of water from the collection site. Thus, total sample size was 30–36 individuals for most species. Sample size ranged from 17 (*Nerita chamaelon*) to 71 (*Littorina littorea*) (Table 1). Sample sizes greater than 36 individuals resulted when HCT determinations were repeated and their results combined (Table 1: *Batillaria sordida*, *Bembicium vittatum*, *Littorina littorea*, *Littorina saxatilis*, *Monodonta labio*, *Morula musiva*). Porous foam plugs blocked tube openings at the tube water surface, keeping individuals immersed. Subsample shell length range was representative of natural adult populations. Subsamples of larger gastropod species were placed in larger test containers (up to 500 ml), allowing freedom of movement, avoiding over-crowding and preventing severe O_2 depletion during determinations, otherwise all other procedures were identical. These larger containers were glass jars with plastic screw caps. After receiving snails, the plastic caps of larger volume jars were screwed on while the jar was submerged in water

TABLE I.

Mean heat coma temperatures (±standard deviation of the mean) for 60 species of intertidal gastropod collected from different rocky shore, mangrove and salt marsh localities at different latitudes and from different shore heights i.e., zonation levels.

Superfamily	Species	Site	Latitude	Habitat	Zonation level	n	Heat coma temp.
Cerithioidea	*Batillaria minima*	Jamaica	18.46° N	Rocky Shore (TP)	Eulittoral Fringe	30	43.5° C ± 0.97
Cerithioidea	*Batillaria multiformis*	Hong Kong	22.37° N	Mangrove	Lower Eulittoral	30	41.3° C ± 0.79
Cerithioidea	*Batillaria sordida*	Hong Kong	22.37° N	Rocky Shore	Lower Eulittoral	42	40.5° C ± 1.63
Cerithioidea	*Cerithidea ornata*	Hong Kong	22.37° N	Mangrove	Eulittoral Fringe	30	43.3° C ± 2.17
Cerithioidea	*Cerithidea rhizophorarum*	Hong Kong	22.37° N	Mangrove	Eulittoral	38	45.5° C ± 1.11
Cerithioidea	*Cerithideopsilla djadjariensis*	Hong Kong	22.37° N	Mangrove	Lower Eulittoral	38	40.9° C ± 1.26
Cerithioidea	*Cerithium antiquata*	Darwin, Australia	12.50° S	Mangrove	Eulittoral Fringe	28	44.1° C ± 0.31
Cerithioidea	*Clypeomorus* sp.	Darwin, Australia	12.50° S	Rocky Shore	Lower Eulittoral	20	40.1° C ± 1.07
Cerithioidea	*Planaxis nucleus*	Jamaica	18.46° N	Rocky Shore	Lower Eulittoral	25	38.1° C ± 0.71
Cerithioidea	*Planaxis sulcatus*	Hong Kong	22.37° N	Rocky Shore	Eulittoral	36	40.8° C ± 1.13
Cerithioidea	*Telescopium telescopium*	Darwin, Australia	12.50° S	Mangrove	Eulittoral	27	44.4° C ± 1.97
Cerithioidea	*Terebralia palustris*	Darwin, Australia	12.50° S	Mangrove	Eulittoral	30	44.7° C ± 0.84
Cerithioidea	*Terebralia semistriata*	Darwin, Australia	12.50° S	Mangrove	Eulittoral	25	43.0° C ± 2.22
Cerithioidea	*Terebralia sulcata*	Hong Kong	22.37° N	Mangrove	Eulittoral	30	43.6° C ± 1.59
Ellobioidea	*Cassidula angulifera*	Darwin, Australia	12.50° S	Mangrove	Eulittoral	20	37.9° C ± 3.39
Ellobioidea	*Cassidula rugata*	Darwin, Australia	12.50° S	Mangrove	Eulittoral	21	39.3° C ± 1.52
Ellobioidea	*Ellobium auriskadae*	Darwin, Australia	12.50° S	Mangrove	Eulittoral	30	39.2° C ± 0.86
Ellobioidea	*Melampus bidentatus*	Woods Hole, Mass	41.54° N	Salt Marsh	Eulittoral Fringe	25	38.8° C ± 1.12
Littorinoidea	*Bembicium vittatum*	Albany, Australia	35.00° S	Rocky Shore	Eulittoral Fringe	42	40.0° C ± 1.64
Littorinoidea	*Cenchritis muricatus*	Jamaica	18.46° N	Rocky Shore	Eulittoral Fringe	28	42.1° C ± 2.13
Littorinoidea	*Littoraria* sp.	Darwin, Australia	12.50° S	Mangrove	Eulittoral Fringe	23	43.9° C ± 0.87
Littorinoidea	*Littoraria filosa*	Darwin, Australia	12.50° S	Mangrove	Eulittoral Fringe	30	42.9° C ± 1.24
Littorinoidea	*Littoraria irrorata*	Port Aransas, Texas	28.84° N	Salt Marsh	Eulittoral Fringe	33	43.7° C ± 1.38
Littorinoidea	*Littoraria melanostoma*	Hong Kong	22.37° N	Mangrove	Eulittoral Fringe	27	44.3° C ± 1.20
Littorinoidea	*Littoraria scabra*	Hong Kong	22.37° N	Mangrove	Eulittoral Fringe	30	43.3° C ± 1.88
Littorinoidea	*Littorina arcana*	Robin Hoods Bay, Eng.	54.61° N	Rocky Shore	Eulittoral Fringe	35	32.8° C ± 0.94
Littorinoidea	*Littorina brevicula*	Hong Kong	22.37° N	Rocky Shore	Eulittoral Fringe	30	40.1° C ± 1.03
Littorinoidea	*Littorina compressa*	Porth Towyn, Wales	53.04° N	Rocky Shore	Eulittoral Fringe	36	27.6° C ± 0.76
Littorinoidea	*Littorina littorea*	Robin Hoods Bay, Eng.	54.61° N	Rocky Shore	Eulittoral	71	32.0° C ± 0.76
Littorinoidea	*Littorina neglecta*	Robin Hoods Bay, Eng	54.61° N	Rocky Shore	Eulittoral	35	31.8° C ± 1.32
Littorinoidea	*Littorina obtusata*	Robin Hoods Bay, Eng.	54.61° N	Rocky Shore	Lower Eulittoral	26	28.3° C ± 2.78
Littorinoidea	*Littorina mariae*	Robin Hoods Bay, Eng.	54.61° N	Rocky Shore	Lower Eulittoral	21	26.4° C ± 2.60
Littorinoidea	*Littorina saxatilis*	Robin Hoods Bay, Eng	54.61° N	Rocky Shore	Eulittoral Fringe	70	32.8° C ± 0.82
Littorinoidea	*Melaraphe neritoides*	Filey Head, Eng.	54.21° N	Rocky Shore	Eulittoral Fringe	21	34.8° ± 1.40
Littorinoidea	*Nodilittorina dilatata*	Jamaica	18.46° N	Rocky Shore	Eulittoral Fringe	30	45.4° C ± 1.59
Littorinoidea	*Nodilittorina exigua*	Hong Kong	22.37° N	Rocky Shore	Eulittoral Fringe	36	44.8° C ± 1.46
Littorinoidea	*Nodilittorina interrupta*	Port Aransas, Texas	28.84° N	Rocky Shore	Eulittoral Fringe	30	44.4° C ± 1.36
Littorinoidea	*Nodilittorina mespillum*	Jamaica	18.46° N	Rocky Shore (TP)	Eulittoral Fringe	30	45.6° C ± 2.19
Littorinoidea	*Nodilittorina pyramidalis*	Hong Kong	22.37° N	Rocky Shore	Eulittoral Fringe	35	46.3° C ± 0.71
Littorinoidea	*Nodilittorina rusca*	Jamaica	18.46° N	Rocky Shore	Eulittoral	30	45.3° C ± 1.68
Littorinoidea	*Nodilittorina unifasciata*	Albany, Australia	35.00° S	Rocky Shore	Eulittoral Fringe	30	41.1° C ± 1.24
Littorinoidea	*Nodilittorina ziczac*	Jamaica	18.46° N	Rocky Shore	Eulittoral Fringe	30	46.9° C ± 2.68
Littorinoidea	*Tectarius antoni*	Jamaica	18.46° N	Rocky Shore	Eulittoral Fringe	27	43.4° C ± 1.65
Muricoidea	*Lepsiella vinosa*	Albany, Australia	35.00° S	Rocky Shore	Eulittoral	30	39.2° C ± 0.96
Muricoidea	*Morula musiva*	Hong Kong	22.37° N	Rocky Shore	Lower Eulittoral	60	39.6° C ± 0.64
Muricoidea	*Nucella lapillus*	Filey Head, Eng.	54.21° N	Rocky Shore	Lower Eulittoral	36	29.6° ± 1.38
Neritoidea	*Nerita atramentosa*	Albany, Australia	35.00° S	Rocky Shore	Eulittoral	30	38.8° C ± 2.14
Neritoidea	*Nerita balteata*	Darwin, Australia	12.50° S	Mangrove	Eulittoral	28	41.1° C ± 1.08
Neritoidea	*Nerita chamaeleon*	Hong Kong	22.37° N	Rocky Shore	Eulittoral Fringe	17	43.3° C ± 0.69
Neritoidea	*Nerita peloronta*	Jamaica	18.46° N	Rocky Shore	Eulittoral	29	40.8° C ± 1.90
Neritoidea	*Nerita tessellata*	Jamaica	18.46° N	Rocky Shore	Eulittoral	30	40.9° C ± 1.20
Neritoidea	*Nerita versicolor*	Jamaica	18.46° N	Rocky Shore	Eulittoral	60	42.3° C ± 1.39
Neritoidea	*Neritina reclivata*	Gulf Shores, AL	30.35° N	Salt Marsh	Lower Eulittoral	30	41.3° ± 0.70
Neritoidea	*Neritina virginea*	Jamaica	18.46° N	Rocky Shore	Eulittoral	30	45.4° C ± 1.16
Neritoidea	*Puperita pupa*	Jamaica	18.46° N	Rocky Shore (TP)	Eulittoral Fringe	30	44.6° C ± 1.81
Onchidioidea	*Onchidium* sp.	Darwin, Australia	12.50° S	Rocky Shore	Lower Eulittoral	30	37.0° C ± 1.20
Trochacea	*Austrocochlea constricta*	Albany, Australia	35.00° S	Rocky Shore	Eulittoral	30	37.5° C ± 1.17
Trochacea	*Austrocochlea concamerata*	Albany, Australia	35.00° S	Rocky Shore	Eulittoral	30	35.6° C ± 0.82
Trochacea	*Lunella coronata*	Hong Kong	22.37° N	Rocky Shore	Lower Eulittoral	30	38.8° C ± 1.02
Trochacea	*Monodonta labio*	Hong Kong	22.37° N	Rocky Shore	Eulittoral	60	37.4° C ± 1.92

TP = Rocky shore species restricted to tide pool habitats

from the collection site in order to exclude all air bubbles and keep individuals immersed.

Containers holding snail subsamples were placed in a water bath to a depth at which bath water level exceeded test tube water level or was equivalent to that in the glass jars. Uniform bath water temperature was maintained by vigorous aeration or a circulating pump. The bath heater switch was manually controlled to raise bath water temperature and, therefore, container water temperature, at a rate of approximately 1°C 5 min^{-1}. This rate of increase makes lag between container water and snail tissue temperatures negligible (Broekhuysen, 1940). Container water temperature was monitored with a thermistor probe extended through the foam test tube plug or a small hole in the jar cap and connected to a YSI model 43-TD tele-thermometer or an eomega 866 Thermometer. Bath water temperature was monitored with a laboratory-grade mercury thermometer or separate thermistor probe.

Each HCT determination began at room temperature (19–26°C, dependent on laboratory ambient temperature). Prior to HCT determination, snails were allowed to attach to container walls and actively locomote. Individuals remaining inactive (i.e., not pedally attaching) during determinations where not included in HCT computations, occasionally reducing sample size below the initial 30 or 36 individuals placed in chambers (Table 1). Number of individuals displaying heat coma symptoms in each container was recorded at every 1°C increase in chamber water temperature. Container water temperature was increased at 1°C 5 min^{-1} until all previously active individuals in all subsamples displayed heat coma symptoms. Thereafter, containers were removed from the bath, partially decanted, and allowed to cool to room temperature. Snails remained in the containers and their recovery observed after a 1–12 h post-treatment return to room temperature (i.e., observed for re-attachment and/or active locomotion).

Included in the data set were HCT determinations for seven species of Jamaican littorinoidean snails (*Cenchritis muricatus, Tectarius antonii, Nodilittorina dilatata, Nodilittorina ziczac, Nodilittorina angustior, Nodilittorina riisei* and *Nodilittorina mespillum*, Table 1) determined by Britton (1992) utilizing the same methodology described above. Across all 60 tested species, HCT was determined for a total of 1,945 individuals.

RESULTS

Correlation of Species' Heat Comma Temperature with Latitude

A high proportion of individuals (> 90–95%) in all tested species recovered from heat coma on cooling to room temperature. For some species, individual subsamples were removed from the bath at sequentially higher temperatures subsequent to entering heat coma. Among these species, lethal acute temperature exposures occurred approximately 3–7°C above HCT, indicating that heat coma is a reversible, nonlethal condition in intertidal gastropods as it is in freshwater gastropods (McMahon 1976, McMahon & Payne 1980).

Mean HCT values among the 60 tested species ranged from a low of 26.4°C for the low-shore littorinoidean, *Littorina mariae*, from Robin Hoods Bay, England (54.61°N) to a high value of 46.9°C for the high-shore littorinoidean, *Nodilittorina ziczac*, from Jamaica (18.4 N) (Table 1). Across all tested individuals, HCT was negatively correlated with latitude (Fig.1A). The relationship between individual HCT and latitude as the controlled variable was best modeled by a quadratic equation:

$$\text{HCT (°C)} = 39.83 + 0.29\ (\text{°Lat}) - 0.0083\ (\text{°Lat}^2)$$
$$(n = 1{,}945,\ \text{d.f.} = 2,\ r^2 = 0.681,$$
$$F = 2071.0,\ P < 0.00001),$$

whose r^2 value indicated that correlation with latitude explained 68% of observed HCT variation (Fig. 1A). Fitting of HCT data for all tested individuals in the superfamily, Littorinoidea (25 species) to a quadratic equation against latitude as the controlled variable yielded the following result:

$$\text{HCT (°C)} = 41.99 + 0.30\ (\text{°Lat}) - 0.0091\ (\text{°Lat}^2)$$
$$(n = 836,\ \text{d.f.} = 2,\ r^2 = 0.855,$$
$$F = 2455.4,\ P < 0.00001),$$

whose r^2 value indicated that correlation with latitude explained 86% of HCT variation (Fig. 1A). Fitting of HCT data for all tested individuals in superfamilies other than the Littorinoidea (35 species) to a quadratic equation against latitude as the controlled variable yielded the following result:

$$\text{HCT (°C)} = 39.59 + 0.25\ (\text{°Lat}) - 0.0078\ (\text{°Lat}^2)$$
$$(n = 1109,\ \text{d.f.} = 2,\ r^2 = 0.409,$$
$$F = 382.75,\ P < 0.00001),$$

whose r^2 value indicated that correlation with latitude explained 41% of HCT variation (Fig. 1). Mean HCT values for littorinoidean species and nonlittorinoidean species fell close to that predicted by quadratic fits of HCT to latitude (Fig. 1A).

Differences in the HCT among Taxonomic Groups

The elevated intercept and slope values of quadratic equations relating HCT to latitude among individuals of littorinoidean and nonlittorinoidean species suggested that, over tested latitudes, littorinoidean species had generally higher HCT than nonlittorinoidean species. This hypothesis was tested by Analysis of Covariance (ANCOVA) of HCT with taxonomic groups as treatments (i.e., Superfamily Littorinoidea versus nonlittorinoidean species) and both latitude of collection and latitude of collection squared as covariates. Use of latitude and latitude squared as covariates in this analysis removed the influence of the negative quadratic relationship with latitude on treatment mean HCT values (see above), allowing the direct effects of the treatments to be statistically assessed. The analysis yielded species' mean HCT values that were "adjusted" to eliminate latitude effects, hereafter referred to as "adjusted mean HCT" values. ANCOVA revealed that the adjusted mean HCT of littorinoidean snails (±95% confidence limits of the mean) (41.1°C ± 0.446, n = 836) was significantly greater than that of nonlittorinoidean species (38.43°C ± 0.209, n = 1,109) (n = 1945, d.f. = 1, F = 401.7, $P < 0.00001$) (Fig. 1B).

ANCOVA with latitude and latitude^2 as covariates also revealed differences in HCT among the seven superfamilies represented in the data set (n = 1945, d.f. = 6, F = 169.6, $P <$ 0.00001). A *post hoc* Scheffe pair-wise comparison test revealed significant differences ($P < 0.05$) among superfamily adjusted mean HCT values (Fig. 2). The Littorinoidea had a higher adjusted mean HCT (42.2°C ± 0.446, n = 836) than the other six superfamilies represented. Adjusted mean HCT of the Cerithoidea (40.8°C ± 0.237, n = 423) and Neritoidea (40.6°C ± 0.277, n = 284) where not different, but were significantly greater than those of the Muricoidea (39.0°C ± 0.808, n = 126), Ellobioidea (38.6°C ± 0.386, n = 96), Trochacea (36.6°C ± 0.287, n = 150) and

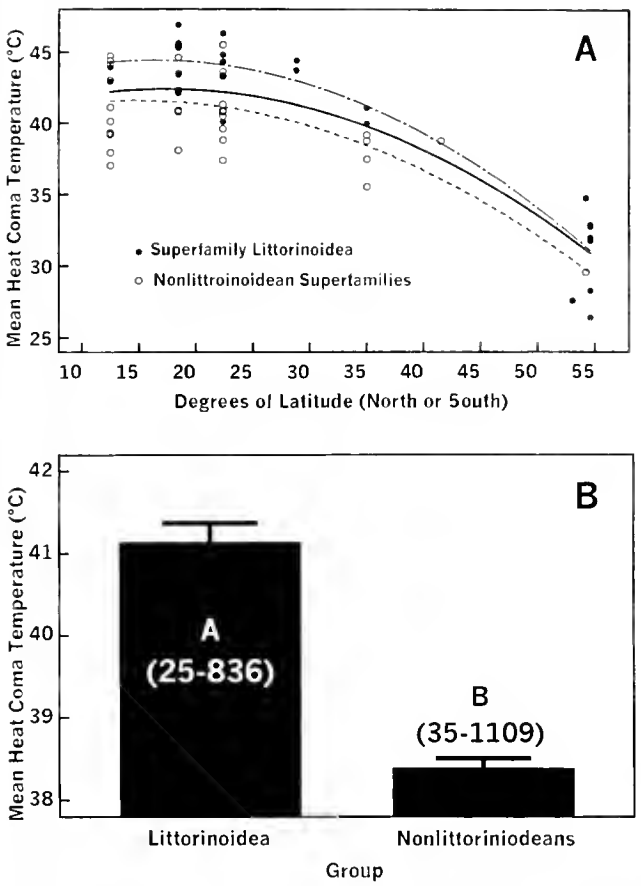

Figure 1. A. Relationship between heat coma temperature (HCT) (vertical axis) and degrees of latitude of collection (horizontal axis) among subsamples of 60 species of intertidal snails. Solid circles represent the mean HCT values for species in the superfamily, Littorinoidea, while open circles are those of species in nonlittorinoidean superfamilies. The solid line represents the best quadratic regression fit of HCT to latitude of collection for all tested individuals, the dot-dashed line, the best fit for littorinoidean snails and the dashed line, the best fit for nonlittorinoidean snails (See Results for regression parameters). B) Adjusted mean heat coma temperatures (HCT) (vertical axis) for littorinoidean and nonlittorinoidean snails. Different letters inside or above histograms indicate significant difference ($P < 0.05$) between means. Numbers within or above the histograms represent the number of species represented in the sample (left of dash) and number of individuals (right of dash) on which means were based. Error bars are 95% confidence limits of the mean.

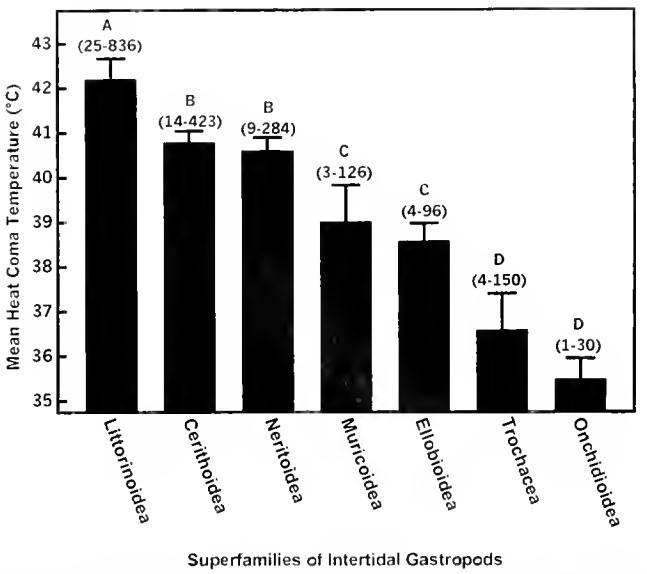

Figure 2. Adjusted mean heat coma temperatures (vertical axis) for snails representing seven different superfamilies of intertidal gastropods (horizontal axis). Vertical bars above histograms represent 95% confidence limits of the mean. Different letters above the histograms indicate significant differences between means ($P < 0.05$). Numbers above the histograms represent the number of species represented in the sample (left of dash) and number of individuals (right of dash) on which means were based.

Onchidioidea (35.5°C ± 0.457, $n = 30$). The adjusted mean HCT of the Muricoidea and Ellobioidea were not different, but were significantly less ($P < 0.05$) than those of the Littorinoidea, Cerithoidea and Neritoidea and greater than those of the Trochacea and Onchidioidea ($P < 0.5$). The adjusted mean HCT of the Trochacea and Onchidioidea did not differ and were significantly lower ($P < 0.05$) than those of all five other superfamilies (Fig. 2).

Differences in HCT among Zonation Levels and Habitat Types

ANCOVA with latitude and latitude^2 as covariates revealed significant differences in adjusted mean HCT of individuals relative to zonation level ($n = 1945$, d.f. = 2, $F = 340.3$, $P < 0.00001$). A *post hoc* Scheffee pair-wise comparison test indicated that the adjusted mean HCT of low-shore (37.5°C ± 0.515, $n = 380$), mid-shore (39.4°C ± 0.320, $n = 750$) and high-shore snails (41.4°C ± 0.362, $n = 815$) differed significantly from each other ($P < 0.05$), thermal tolerance increasing with zonation level (Fig. 3A).

ANCOVA with latitude and latitude^2 as covariates also revealed significant differences in adjusted mean HCT of individuals relative to habitat (n = 1945, d.f. = 2, $F = 23.9$, $P < 0.00001$). A *post hoc* Scheffee pair-wise comparison test indicated that the adjusted mean HCT of rocky shore (40.2°C ± 0.292, $n = 1,370$), mangrove (41.1°C ± 0.228, $n = 479$) and salt marsh snails (41.7°C ± 0.486, $n = 88$) all differed significantly ($P < 0.05$), rocky-shore snails being least thermally tolerant and salt-marsh snails, most tolerant (Fig. 3B).

A multiple factor ANCOVA with latitude and latitude^2 as covariates and zonation level and taxonomic group (i.e., littorinoidean versus nonlittorinoidean snails) as treatments, revealed significant differences in the adjusted mean HCT of snails by zonation level (d.f. = 2, $F = 154.1$, $P < 0.00001$) and taxonomic group (d.f. = 1, $F = 23.5$, $P < 0.00001$) with significant interaction between treatments (d.f. = 2, F = 48.9, $P < 0.00001$). Adjusted mean HCT values and 95% confidence limits for each treatment combination were: low-shore littorinoideans, 35.1°C ±0.832, $n = 47$; mid-shore littorinoideans, 39.8°C ±0.963, $n = 136$; high-shore littorinoideans, 39.4°C ±0.463, $n = 653$; low-shore nonlittorinoideans, 35.5°C ±0.389, $n = 333$; mid-shore nonlittorinoideans, 36.7°C ±0.259, $n = 614$; and high-shore nonlittorinoideans, 39.2°C ±0.362, $n = 162$ (Fig. 4A). *Post hoc* Scheffe pair-wise comparisons indicated that adjusted mean HCT for all snails significantly increased ($P < 0.05$) with zonation level (i.e., across low-shore, mid-shore and high-shore zones) (Fig. 4A). Adjusted mean HCT did not differ among littorinoidean and nonlittorinoidean snails in either the low-shore or high-shore zones, but

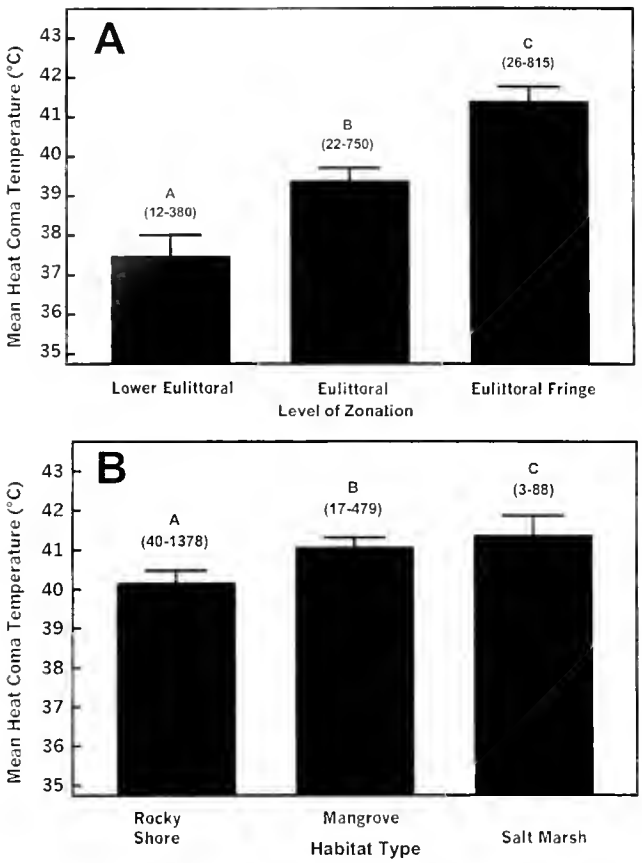

Figure 3. (A) Adjusted mean heat coma temperatures (vertical axis) for intertidal snails relative to zonation level (horizontal axis). (B) Adjusted mean heat coma temperatures (vertical axis) for intertidal snails relative to habitat type (horizontal axis). In both figures, vertical bars above histograms represent 95% confidence limits of the mean. Different letters above the histograms indicate significant differences between means ($P < 0.05$). Numbers above the histograms represent the number of species represented in the sample (left of dash) and number of individuals (right of dash) on which means were based.

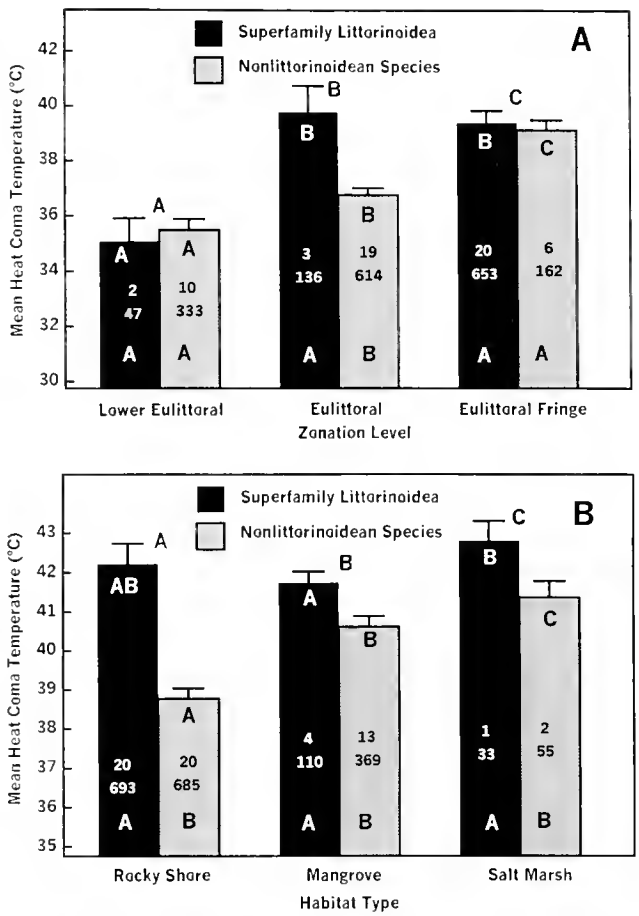

Figure 4. (A) Adjusted mean heat coma temperatures (HCT) (vertical axis) for intertidal snails relative to zonation level (horizontal axis) among individuals within (black histograms) and without (gray histograms) the superfamily Littorinoidea. (B) Adjusted mean heat coma temperatures (vertical axis) for intertidal snails relative to habitat type (horizontal axis) among individuals within (black histograms) and without (gray histograms) the superfamily Littorinoidea. For both figures, vertical bars above histograms represent 95% confidence limits of the mean. Different letters at the base of the histograms indicate significant differences between mean HCT ($P < 0.05$) for littorinoidean versus nonlittorinoidean snails within a specific habitat. Different letters near the top of the histograms indicate significant differences between mean HCT ($P < 0.05$) for littorinoidean and nonlittorinoidean snails, respectively, across different habitats ($P < 0.05$). Different letters above pairs of histograms for littorinoidean versus nonlittorinoidean snails indicate significant differences in the combined mean HCT for both groups across different habitats. Numbers within the histograms represent the number of species represented in the sample (upper number) and number of individuals (lower number) on which the means were based.

was significantly greater ($P < 0.05$) among littorinoidean versus nonlittorinoidean mid-shore snails. Within the Littorinoidea, adjusted mean HCT was significantly lower in low-shore snails ($P < 0.05$) relative to mid-shore or high-shore species among which adjusted mean HCT did not differ. Among nonlittorinoidean snails, adjusted HCT increased significantly ($P < 0.05$) across all zonation levels (Fig. 4A).

A multiple factor ANCOVA with latitude and latitude^2 as covariates and habitat type and taxonomic group (i.e., Littorinoidea versus nonlittorinoideans) as treatments revealed significant differences in the adjusted mean HCT of snails among different habitats (d.f. = 2, $F = 21.4$, $P < 0.00001$) and taxonomic groups (d.f. = 1, $F = 89.2$, $P < 0.00001$) with significant treatment interaction (d.f. = 2, $F = 29.9$, $P < 0.00001$). Adjusted mean HCT values and 95% confidence limits for each treatment combination were: littorinoideans on rocky shores (42.2°C ± 0.508, $n = 693$), littorinoideans in mangroves (41.7°C ± 0.277, $n = 110$), littorinoideans in salt marshes (42.8°C ± 0.495 $n = 33$), nonlittorinoideans on rocky shores (38.8°C ± 0.275 $n = 685$), nonlittorinoideans in mangroves (40.6°C ± 0.279, $n = 369$), nonlittorinoideans in salt marshes (41.4°C ± 0.423, $n = 55$) (Fig. 4B). A *post hoc* Scheffe pair-wise comparison test indicated that adjusted mean HCT for all snails differed significantly ($P < 0.05$) among habitats, with rocky shore species having the lowest adjusted mean HCT, mangrove species having intermediate HCT and salt marsh species having the highest HCT (Fig. 4B). In all three habitats, littorinoidean species had significantly higher ($P < 0.05$) adjusted mean HCT values than nonlittorinoidean species. Within the Littorinoidea, adjusted mean HCT of rocky shore snails was not different from that of either mangrove or salt-marsh snails, however that of mangrove species was significantly lower than that of salt-marsh species ($P < 0.05$) (Fig. 4B). In contrast, adjusted mean HCT significantly varied ($P < 0.05$) among nonlittorinoidean

species in all three habitats, being least in rocky shore species, intermediate in mangrove species and greatest in salt marsh species (Fig. 4B).

DISCUSSION

Review of the literature indicates that the thermal tolerance of intertidal invertebrates tends to increase with decreasing latitude, species adapting to warmer, lower-latitude habitats by evolving increased tolerance (Vernberg & Vernberg 1972). Only two studies have examined intertidal gastropod upper thermal limits comparatively across geographically separate areas. Tolerance times on chronic exposure to lethal temperatures increased in intertidal gastropod species from lower latitudes among geographic areas including: Woods Hole, Massachusetts (41.5°N); Ocean Springs, Mississippi (30.4°N); and Bimini, Bahamas (24.9°N) (Fraenkel 1968). In contrast, intertidal snail acute upper thermal limits were 0.5–3°C higher at the higher latitude of Hong Kong (22.4°N) compared to that of snails from the lower latitude of Dar es Salaam, Tanzania (8.6°S) (Stirling, 1982). Such conflicting data either results from examination of too few geographic sites and/or relatively narrow latitudinal ranges. Further, use of different thermal tolerance determination techniques by different investigators prevents direct comparison of data across latitudes. In the present study, the same technique for determination of acute thermal tolerance was applied across 60 species from 10 geographically separate sites over a broad latitudinal range of 42.1° (i.e., 12.5–54.6°) encompassing temperate, subtropical and tropical shores. The resulting data indicated that, while species' HCT increased dramatically from temperate to subtropical shores (25–55° latitude), it did not further increase below 25°, resulting in the negative quadratic relationship between HCT and latitude depicted in Fig. 1A. Interestingly, the 25° latitude threshold below which species' HCT stabilized lies quite close to the 23.45° north and south latitudes of the Tropic of Cancer and Tropic of Capricorn, demarcating the tropics.

Within the tropics, temperatures are maximal and annual temperature ranges, minimal, compared to subtropical and temperate habitats. Accordingly, thermal selection pressures in the tropics should not vary latitudinally while outside of the tropics it should decrease with increasing latitude. Thus, Fraenkel (1968) recorded increasing thermal tolerance with decreasing latitude among snails on temperate and subtropical shores (24.9–41.5°N) while Stirling (1982) did not find a similar trend on two tropical shores. Alternately, the thermal tolerance of tropical intertidal snails may lie close to the physiological maximum for gastropods, making it unresponsive to latitudinally mediated thermal selective pressures within tropical latitudes. The acute upper thermal limits of tropical intertidal snails recorded in this study lie near the maxima recorded among the majority of invertebrates with the exception of species restricted to atypically warm habitats such as hot springs (Precht et al. 1973).

Littorinoidean snails have generally been reported to be most thermally tolerant taxon in previous comparative studies of gastropod thermal tolerance on rocky shores (Broekhuysen 1940, Evans 1948, Southward 1958, Fraenkel 1966 and 1968, Stirling 1982, Cleland & McMahon 1990, McMahon 1990, Britton 1992), in mangroves (McMahon & Britton 1985) and in salt marshes (Table 1, see mean HCT values for *Littoraria irrorata*, *Melampus bidentatus*, *Neritina reclivata*). This observation was confirmed by this study in which the adjusted mean HCT for littorinoidean snails was 2.7°C greater than that of nonlittorinoidean snails. Further, the adjusted mean HCT of littorinoidean snails at 42.2°C was significantly greater than those of snails in the six other superfamilies tested (range = 35.5–40.6°C) (Fig. 2). McMahon (1990) hypothesized that the elevated upper thermal limits of intertidal littorinoideans relative to other intertidal snail taxa reduces their dependence on evaporative cooling to maintain body temperatures below critical thresholds during insolation, thus, maximizing their tolerance of emersion by minimizing water loss rates. Thus, the high temperature tolerance of littorinoideans, confirmed in this study, may contribute significantly to their world-wide dominance of the upper eulittoral fringe - maritime zones of rocky shores and mangroves. Indeed, both laboratory (McMahon 1990, McMahon & Yipp 1992) and field studies (Lewis 1963, Vermeij 1971) indicate that high-shore gastropods, particularly littorinoideans, appear to be much less dependent on evaporative cooling to regulate body temperature when emersed in elevated air temperatures than mid- and low-shore species. Interestingly, snails in the superfamilies, Cerithoidea and Neritoidea, had insignificantly different adjusted mean HCT values of 40.8°C and 40.6°C, respectively, which, while significantly lower than those of the Littorinoidea, were significantly greater than the adjusted mean HCT of snails in the other four tested superfamilies (Fig. 2). Along with the Littorinoidea, the Cerithoidea and Neritoidea make up the majority of upper eulittoral fringe-maritime zone species in rocky shore and mangrove habitats, suggesting that elevated thermal tolerance is a universal physiological adaptation among high-shore gastropod species.

The observation that thermal tolerance increases with increasing shore height in intertidal gastropods (Underwood 1979, McMahon 1990) is strongly supported by this study. Thermal tolerance increased significantly with zonation level, low-shore, mid-shore and high-shore snails displaying a progressive increase in adjusted mean HCT of 37.5°, 39.4°, and 41.4°C, respectively (Fig. 3A). Increased thermal tolerance among mid- and high-shore species appears to be a resistance adaption to rapid tissue heating by insolation during tidal excursions. Insolation heats snail tissues equivalently within 30 minutes emersion into direct sunlight in either region (Southward 1958, Grainger 1969). Thus, the higher thermal tolerance of high-shore species reduces reliance on evaporative cooling, increasing emersion tolerance by minimizing water loss (McMahon 1990, McMahon & Yipp 1992, see previous). This pattern of thermal tolerance increasing with zonation level was maintained in both littorinoidean and nonlittorinoidean snails. Among nonlittorinoidean species, adjusted mean HCT was 35.5°, 36.7° and 39.2°C for low-, mid- and high-shore snails, respectively (Fig. 4A), indicative of increasing selective pressure for thermal tolerance with shore height. A different vertical tolerance pattern emerged among littorinoidean snails in which adjusted mean HCT values of mid- and high-shore snails were not different at 39.8° and 39.4°C, respectively, but were both greater than that of low-shore snails at 35.1°C (Fig 4A). Mid- and low-shore species in the subfamily Littorininae appear to have evolved from ancestors occupying the upper eulittoral fringe - maritime zone (Reid 1989, 1996). The results of this research support this hypothesis. Eulittoral littorinoidean species appear to have retained the elevated thermal tolerance characteristic of their high-shore ancestors, suggesting that there may be little selective pressure for its reduction among species evolving from high shore into mid-shore habits. Indeed, the extreme temperatures experienced during insolation may be similar in mid- and high-shore habitats, with only the duration of exposure differing between them (Southward 1958,

Grainger 1969). Thus, among mid-shore species, the adjusted mean HCT of littorinoidean snails (39.8°C) was significantly greater than that of nonlittorinoideans (36.7°C) (Fig. 4A). In contrast, invasion of thermally stable low-shore habitats appears to have resulted in evolution of a reduced thermal tolerance among low-shore littorinoidean species, their adjusted mean HCT (35.1°C) being insignificantly different from that of low-shore nonlittorinoidean species (35.5°C) (Fig. 4A).

There were also significant differences in adjusted mean HCT relative to habitat. That of rocky-shore snails was lowest at 40.2°C, intermediate in mangrove snails at 41.1°C and greatest in salt-marsh snails at 41.7°C (Fig. 3B). While the biological significance of this 1.5°C HCT range among these habitats is debatable, much clearer patterns emerged when differences were separately analyzed among littorinoidean and nonlittorinoidean snails. Adjusted mean HCT was similar among littorinoidean snails from rocky shore, mangrove and salt marsh habitats, ranging from 41.7°C (mangrove) to 42.8°C (salt marsh) (Fig. 4B). There appears to be little selective advantage in the 1.1°C HCT range among these habitats. The relatively minor habitat differences in the thermal tolerance of littorinoidean snails may again reflect their evolutionary history with the majority of mangrove and salt-marsh littorinoidean species likely to have evolved from temperature tolerant high rocky-shore ancestors (Reid 1989). Therefore, it appears that the elevated thermal tolerance of primitive, ancestral, high rocky-shore littorinoideans may have been retained by species evolving from them into mangrove and salt-marsh habitats, reflected by the significantly elevated adjusted mean HCT of littorinoidean relative to nonlittorinoidean snails in all three habitats (Fig. 4B).

In contrast to littorinoidean snails, there were clear habitat differences in the adjusted mean HCT of nonlittorinoidean species. Among nonlittorinoideans, rocky-shore snails had the lowest adjusted mean HCT (38.8°C), with that of mangrove snails being intermediate (40.6°C) and that of salt-marsh snails being greatest (41.4°C), yielding an adjusted mean HCT habitat range of 2.6°C (Fig. 4B). This result suggests that there may be differences in thermal selection pressures among the three tested habitats for nonlittorinoidean snails. Unlike littorinoidean species, the majority nonlittorinoidean intertidal taxa may have radiated up the shore from less thermally tolerant low-shore species, evolution of increased thermal tolerance allowing penetration of mid- and high-shore habitats (McMahon 1990). Rocky shores, particularly boulder shores, offer microrefugia from insolation and thermal stress on vertical rock surfaces, in crevices and beneath boulders. There are much fewer refugia from thermal stress in mangals, particularly among species inhabiting mangrove tree trunks or leaves (McMahon & Britton 1985) perhaps accounting for their greater mean HCT relative to rocky-shore species. However, mangrove snails are generally protected from insolation by the mangal canopy. In contrast, mid-shore and high-shore salt-marsh snails generally inhabit the stems of marsh grasses (McMahon and Russell-Hunter 1981, McMahon 1992), where they are exposed to intense insolation and have no access to thermal refugia. Thus, the increased thermal tolerance of nonlittorinoidean snails from mangrove and salt marsh habitats may have been evolved in response to increased thermal selection pressures and reduced access to thermal refugia in these habitats relative to generally more heterogeneous rocky shores.

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LAGOONAL LITTORINIDS: SHELL SHAPE AND SPECIATION

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ABSTRACT Variables related to shell shape have been measured in littorinids from brackish lagoons and coastal sites. After removal of size related effects, the data were analyzed using multivariate techniques. On Canonical Variate 1 there was good separation of the shells of the lagoonal animals from those of animals from the coast and a tidal lagoon. The former, for example, had lighter, and therefore thinner, shells for any given shell size and a smaller jugosity of the aperture lip. The lagoonal shells from Golam Head and the coastal animals from Robin Hood's Bay could each be separated clearly from the other samples. Although there are clear morphometric differences in the shells, it is not possible without appropriate breeding experiments to raise the lagoonal animals from *L. saxatilis* var. *lagunae* (*L. tenebrosa*) to species status. The importance of conserving lagoonal habitats is considered in terms of the preservation of biodiversity.

KEY WORDS: Littorinids, brackish lagoons, *Littorina saxatilis* var. lagunae

INTRODUCTION

There are three clearly recognizable taxa of rough periwinkles on European shores, the ovoviviparous *Littorina saxatilis* (Olivi), and the oviparous *L. arcana* Hannaford Ellis, and *L. compressa* Jeffreys. The last two taxa are non-contentious with regard to their species status. However, *L. saxatilis* has a wide range of habitats and includes populations of differing shell morphology some of which are found in discrete environments. This has led to various attempts to separate taxa from within this complex. Two of these are worthy of further investigation, i.e. *L. neglecta* Bean, which is found in the barnacle zone living sympatrically with "normal" *L. saxatilis* (*L. saxatilis* B) (Grahame et al. 1995, Hull et al. 1999), and *L. tenebrosa* (Montagu) which occurs in brackish lagoons (Barnes 1993). Two other forms within *L. saxatilis*, H and M, have also been recognized (Hull et al. 1996).

The subject of this paper is on littorinids that inhabit brackish lagoons. There are two problems. Firstly, the use of "*tenebrosa*" is confusing and is hence probably inappropriate (Barnes 1993). In the past it has been used in a very broad sense to include any littorinid with a high-spired shell occurring in sheltered locations including lagoons (Forbes & Hanley 1853). It has also been used in a much more restricted way to include only those littorinids which (a) have a small (usually <6 mm high), very fragile, smooth, plumply-whorled shell which is black or brown and often reticulated, and (b) live permanently submerged on macrophytes (such as *Chaetomorpha*) in brackish lagoons (Muus 1967).

Secondly, the situation is compounded by the presence in some lagoons of littorinids which fit the above description of *L. tenebrosa* whereas in others there are animals which live on the substrate, fit the wider definition of Forbes & Hanley (1853) and may be referred to as *L. saxatilis s.s.* (e.g. Barnes 1987). Furthermore, both forms have been reported as occurring in the same lagoon in some instances (Smith 1982), although Barnes (1993) was only able to find *L. saxatilis s.s.* in the Fleet, Dorset and Cemlyn Lagoon, Anglesey, at which sites *L. tenebrosa* had also previously been reported (Seaward 1980; Barnes 1987).

Muus (1967) and Smith (1982) suggested that the "*tenebrosa*" animals are probably a distinct species, whereas Barnes (1993) concluded that, on the basis of shell variables, this is not the case and that, although the two forms appear to be reproductively isolated, they should be referred to as *L. saxatilis* var. *lagunae*. Reid (1996) also could find no case for species status for this form. In a preliminary study on five allozyme loci, Gosling et al. (1998) concluded that the two forms are genetically differentiated. However, in a more detailed investigation of 12 polymorphic enzyme loci, Wilson et al. (1999) found no allele unique to either form, and concluded that, although there is a barrier to gene flow between them, they are not distinct species.

Barnes' (1993) conclusions were based on five measurements of shell variables and two of operculum variables. The present study extends the number of measured variables and the number of populations in an attempt to clarify the situation, particularly since ecological barriers can result in populations diverging to species status in spite of close similarities at the molecular level (Morell 1999).

MATERIAL AND METHODS

Samples of lagoonal littorinids were obtained from both tidal and isolated habitats (Table 1, Fig. 1). In eastern England 28 lagoons at nine sites were visited. Littorinids were found in only five of these, representing four sites. They were also found in the Fleet in southern England and at Golam Head in the west of Ireland.

The Fleet is a tidal lagoon, open at its eastern end to the English Channel; the sample was taken from gravel on the seaward side of the lagoon near its eastern end (East Fleet). At Golam Head the sample was taken from the landward end of the lagoon near a small freshwater inlet and about 100 m from the seaward end. The animals were completely submerged on the alga *Chaetomorpha*. At its seaward end there are rocks which are continuous with those of the adjacent shore. At Alderton the lagoon is surrounded by shingle on its seaward side and is separated from the sea by a shingle dune about 6 m high. The animals occurred over a short stretch on this side; they were found on small stones near and at the water's edge and on the surface of the mud. Lagoonal littorinids were found in two lagoons at Cley which again were separated

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TABLE 1.

Lagoonal and coastal sites.

Location	National Grid	N
Isolated lagoons		
Alderton, Suffolk, England	[1]TM 363419	53
Cley Eye, Norfolk, England	[1]TG 067447	50
Holkham Hole, Norfolk, England	[1]TF 886451	72
Snettisham (Shepherd's Port), Norfolk, England	[1]TF 649319	32
Lagoon with occasional incursions of sea water		
Golam Head, Galway, Ireland	[2]L 826214	54
Tidal lagoon		
The Fleet (east end), Dorset, England	[1]SY 664757	53
Open coast		
Golam Head, Galway, Ireland	[2]L 826214	38
Robin Hood's Bay, Yorkshire, England	[1]NZ 957058	50
Wells-next-the-sea, Norfolk, England	[1]TF 915456	50

[1] British National Grid; [2] Irish National Grid.

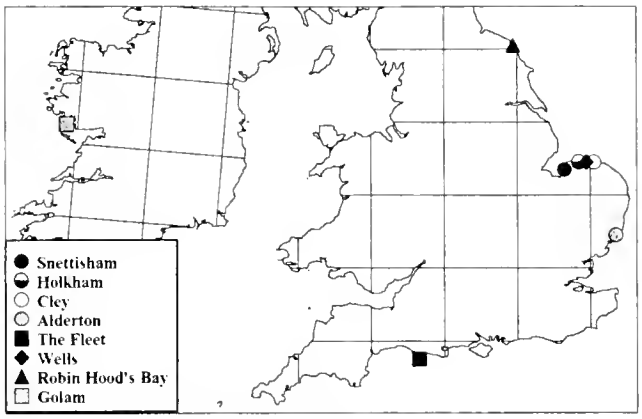

Figure 1. Location of sampling sites in Britain and Ireland.

from the sea by a gravel dune about 7 m in height. They occurred both on algal (*Chaetomorpha*) mats and on stones. At Holkham the lagoon was on the landward side of a very mature dune about 8 m in height covered in trees (Scot's Pine, Holm Oak, and Birch) and bushes. Similarly, the lagoon itself was largely surrounded by trees and bushes. The animals were found on the seaward side of the lagoon completely submerged on the alga *Chaetomorpha*, although some were also found on submerged wood. At Snettisham the lagoon was separated from the sea by a mature sand dune about 6 m in height. The animals were found completely submerged on stones well away from the edges of the lagoon.

At Golam Head tidal incursions occur on spring tides (Wilson et al. 1999). At Snettisham, sea water incursions are unlikely and would certainly be rare; at Alderton and, particularly, at Cley they would probably be even less likely, while at Holkham the only possible connection with sea water would be underground through the substrate. All of the lagoonal sites except Holkham were open and devoid of tall vegetation in their immediate vicinity. There were no coastal sites with littorinids in the close proximity of Alderton, Cley or Snettisham; the nearest coastal site to Holkham is 2.35 km away at Wells-next-the-sea. The condosity of the water was checked at Alderton and Cley and was in excess of 85% seawater.

Other samples were taken from rocky shores on the coast or, in the case of Golam Head, at the entrance to a lagoon (Table 1, Fig. 1). The sample from Golam Head was taken at the seaward end of the lagoon where the rocky shore merged with the edge of the lagoon, providing a very sheltered habitat. This was about 100 m from the site where the lagoonal sample was collected. The sample from Wells-next-the-sea was the closest site to the lagoon at Holkham (2.35 km). Robin Hood's Bay was chosen as representing a typical, somewhat sheltered, east coast boulder site.

The conventional measurements used in previous studies of

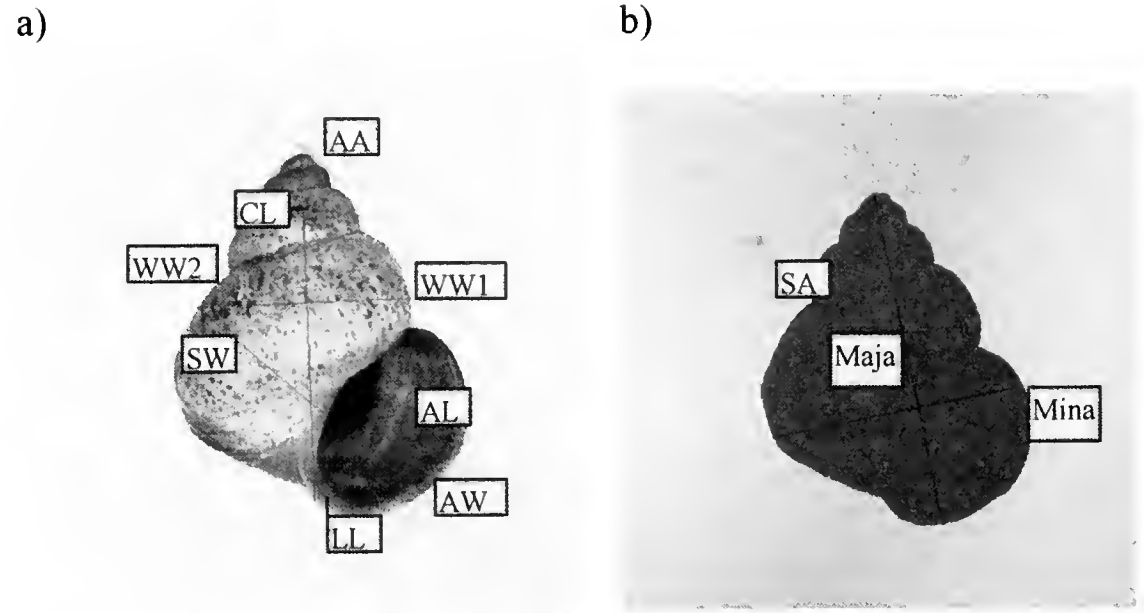

Figure 2. The measurements made on (a) the shell profile and (b) the shell silhouette. AA, apical angle, AL, aperture length (excluding the lip); AW, aperture width; CL, columella length; LL, lip length; Maja, major axis; Mina, minor axis; SA, surface area (in profile); SW, shell width (excluding aperture width); WW1, whorl width 1 (width of whorl at right angles to the columella axis); WW2, whorl width 2 (across the suture between the first and second whorls). In addition shell weight was measured.

littorinids were made, i.e. columella length, lip length, aperture length, width of first whorl at right angle to the columella axis, width of shell minus the aperture, aperture width, width of the suture between the first two whorls and the apical angle (Grahame et al. 1995). Three additional measurements were made, i.e. major axis (maximum linear dimension), minor axis (maximum linear dimension at right angles to the major axis) and shell profile area (Fig. 2). The shells were also weighed.

Canonical Discriminant Analysis, Principal Component Analysis, Factor Analysis and Discriminant Analysis were all carried out on the data after removing the effects of size by standardizing using the geometric mean (except of course for apical angle). Discriminant analysis was also carried out on the raw data.

RESULTS

The animals from Holkham and Golam Head were identified provisionally as *L. tenebrosa* on the basis that they lived permanently submerged on *Chaetomorpha*, their shells were less than 8 mm high and, particularly in the case of those from Holkham, were plumply whorled (Fig. 3a, b). Animals from East Fleet, Alderton and Cley possessed shells which were much more pointed (Fig. 3d-f); those from East Fleet reached 9 mm, while those from Alderton and Cley reached about 13 mm and 14 mm in height respectively; they were not permanently submerged. The shells of animals from Snettisham were somewhat intermediate (Fig. 3c); they were rather less bulbous than the Golam and Holkham animals, were found on stones not algae, but were permanently submerged and were less than 7 mm in height.

The shells of the coastal animals from Golam, Wells and Robin Hood's Bay were all fairly pointed and had a clear jugosity (relative aperture lip length) (Fig. 3g-i). Of the lagoonal animals, only the shells of those from East Fleet had anywhere near the same degree of jugosity. The Robin Hood's Bay shells reached a height of about 12 mm; those from Golam and Wells reached about 16 mm.

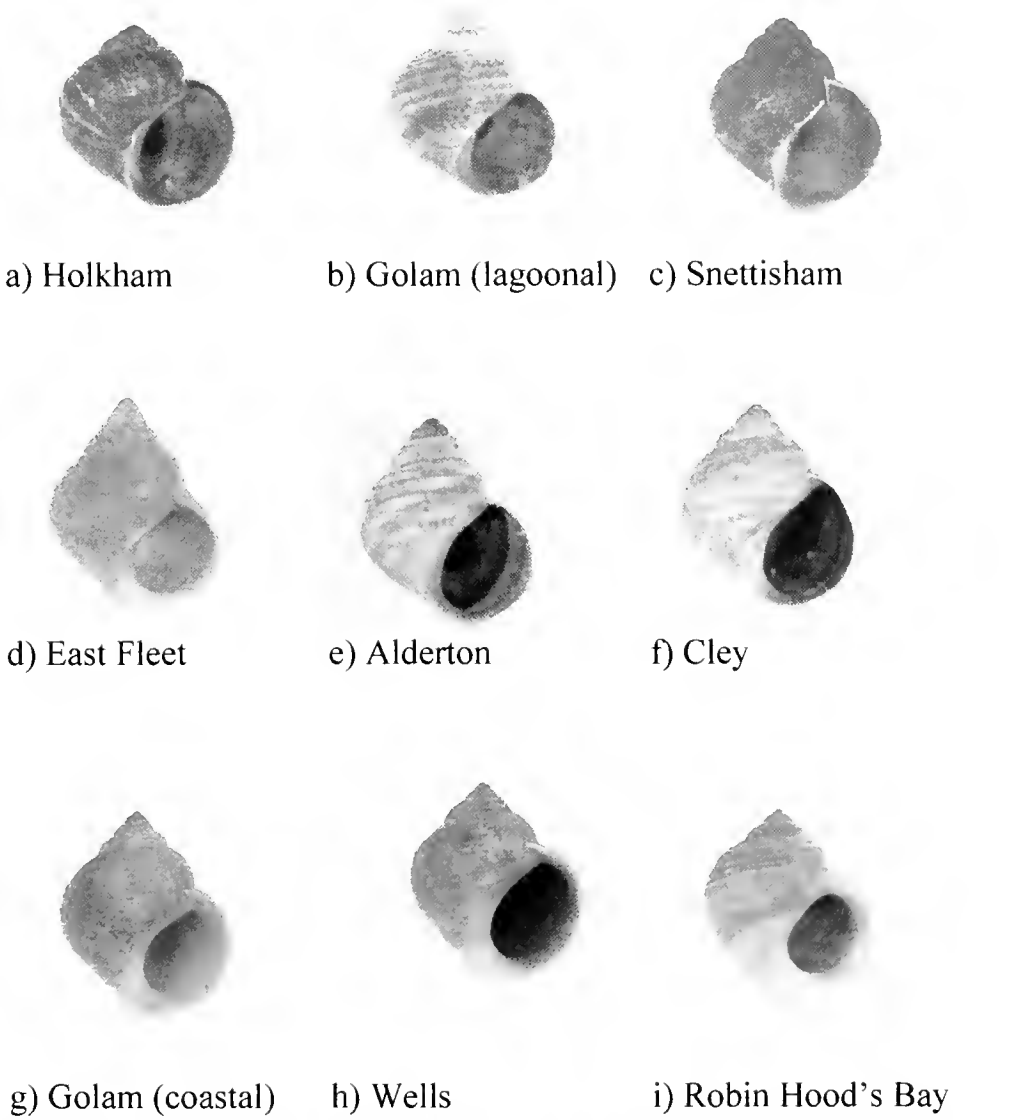

Figure 3. Profiles of shells from the localities stated. All shells are adjusted to the same overall width. Actual columella lengths are: (a) 5.1 mm, (b) 5.1 mm, (c) 5.5 mm, (d) 6.5 mm, (e) 7.2 mm, (f) 6.3 mm, (g) 11.5 mm, (h) 9.0 mm, (i) 9.2 mm.

TABLE 2.

Canonical Discriminant Analysis of the data after removing the effects of size by standardizing using the geometric mean.

	CAN1 51%	CAN2 23%	CAN3 18%
LL	0.9294	−0.2868	0.0811
WEIGHT	0.7228	0.1489	0.1981
SW	−0.0964	0.6524	0.4566
AA	−0.1989	−0.4188	0.6534
CL	−0.2341	0.4524	−0.2457
MINA	−0.4030	0.1948	0.4910
WW2	−0.4707	*0.6520	−0.4328
AL	−0.5671	0.1604	0.3739
AW	−0.6996	−0.4945	0.2274
MAJA	−0.7367	0.3475	0.0215
WW1	−0.7956	0.0083	−0.1916
SA	−0.8302	0.1815	0.4626

Dark shading, highest positive values; *light shading*, highest negative values.
* Not a high value using Principal Component Analysis and Factor Analysis.

Canonical Discriminant Analysis indicated that 51% of the variation was attributable to CAN 1, with lip length and shell weight being opposed to surface area, whorl width 1 and major axis length (Table 2). Twenty three percent of the variation was attributable to CAN2, with whorl width 2 and shell width opposed to aperture width and apical angle, and 18% to CAN3, with apical angle opposed to whorl width 2. Principal Component Analysis and Factor Analysis yielded broadly similar results, except that the second and third factors of the Canonical Discriminant Analysis were reversed in order of importance in the other two analyses. However, Whorl Width 2 in PRIN 3 and FACTOR 3 (corresponding to CAN2) was of minor importance. Some of the important relationships revealed by these analyses were explored further.

When shell weight was plotted against major axis length (see CAN1) the regression slopes fell into two clear groups, the shells from the coastal samples, together with those from East Fleet, having steeper slopes than the other (lagoonal) shells (Fig. 4a). Pooling the data from the two groups gave regression lines with a slope of 0.0448 for the lagoonal shells and 0.0604 for the coastal + East Fleet shells (Fig. 4b). There was virtually no overlap between the two sets of data points and the Spearman Rank Correlation Coefficient (r_s) was ≥0.977 in both cases. A plot of lip length against major axis length (see CAN1) separated clearly the coastal samples from the others; for any given major axis length the former always had a longer lip (i.e. greater jugosity). The East Fleet shells were confirmed as having lip lengths intermediate between those of coastal and the other lagoonal samples (Fig. 5a).

Plotting aperture width against shell width (see CAN2) did not separate the samples entirely along coastal versus lagoonal lines. Robin Hood's Bay shells were more similar to those of lagoonal animals, except for lagoonal Golam shells that were grouped with those from East Fleet, coastal Golam, and Wells (Fig. 5b).

The shells from Holkham, Snettisham, Golam (lagoonal) and Robin Hood's Bay had the most obtuse spires, the angle decreasing with increase in Whorl Width 2 (see CAN3). Those from East Fleet were noticeably the most pointed but the angle increased with increase in Whorl Width 2. Thus the separation was again not strictly coastal versus lagoonal (Fig. 5c).

CAN 1 expressed most clearly the separation of coastal and lagoonal animals and this is seen when the three canonical variates

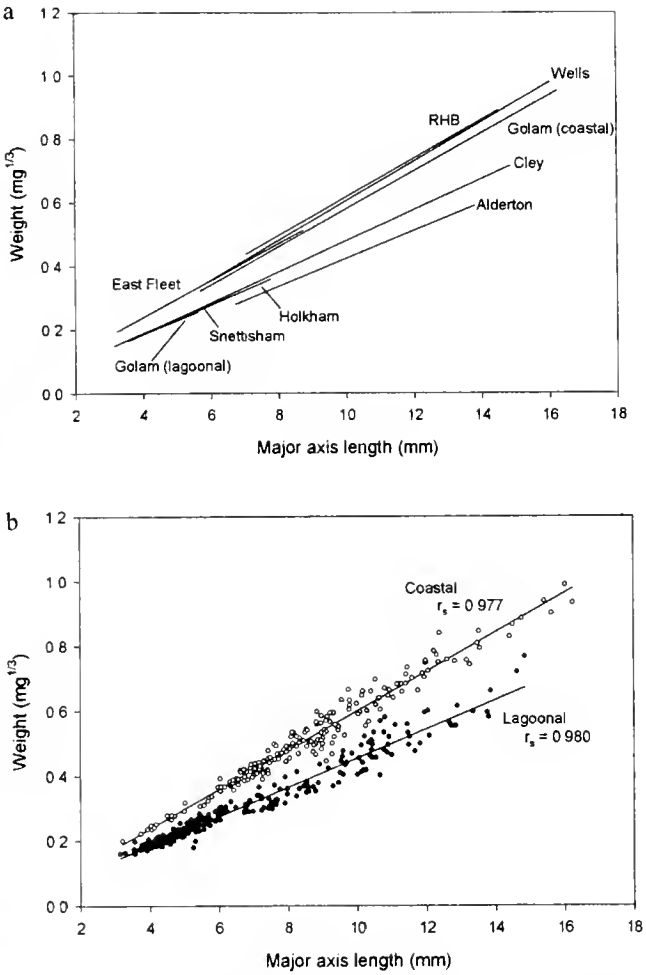

Figure 4. (a) Shell weight ($mg^{1/3}$) plotted against major axis length (mm). Regression lines for each of the nine populations. RHB, Robin Hood's Bay. (b) Shell weight ($mg^{1/3}$) plotted against major axis length (mm). Plots of individual measurements and regression lines for the pooled data. Open circles, coastal sites + East Fleet; filled circles, other lagoonal sites.

are plotted against each other (Fig. 6a). The shells from East Fleet, however, fall into the "coastal" group and indeed there is considerable overlap between them and those from Golam (coastal) and Wells. The coastal shells separate out along the CAN2 and CAN3 axes in the order East Fleet, coastal Golam, Wells and Robin Hood's Bay. The Robin Hood's Bay shells separate out almost completely from the above three samples; the box surrounding the cluster of 50 shells from this site contains only one other shell (from Wells) (Fig. 6a). Amongst the lagoonal shells, the only group that could be separated by rotation of the axes was that from Golam (lagoonal), along the CAN2 and CAN3 axes (Fig. 6b); the box surrounding the cluster of 54 shells contains only one other shell (from Alderton).

Finally a Discriminant Analysis was carried out on the size-adjusted data (Table 3). As expected from the above, the most consistent samples were those from Robin Hood's Bay and Golam (lagoonal), where 98% and 94% of the shells respectively were self classifying. Except for East Fleet, no misclassifications occurred between lagoonal and coastal shells. The shells from Cley showed the greatest degree of misclassification, with 20% classifying to Alderton, 12% to Holkham and 8% to Snettisham. Snettisham shells also had a high proportion of misclassifications, with 19%

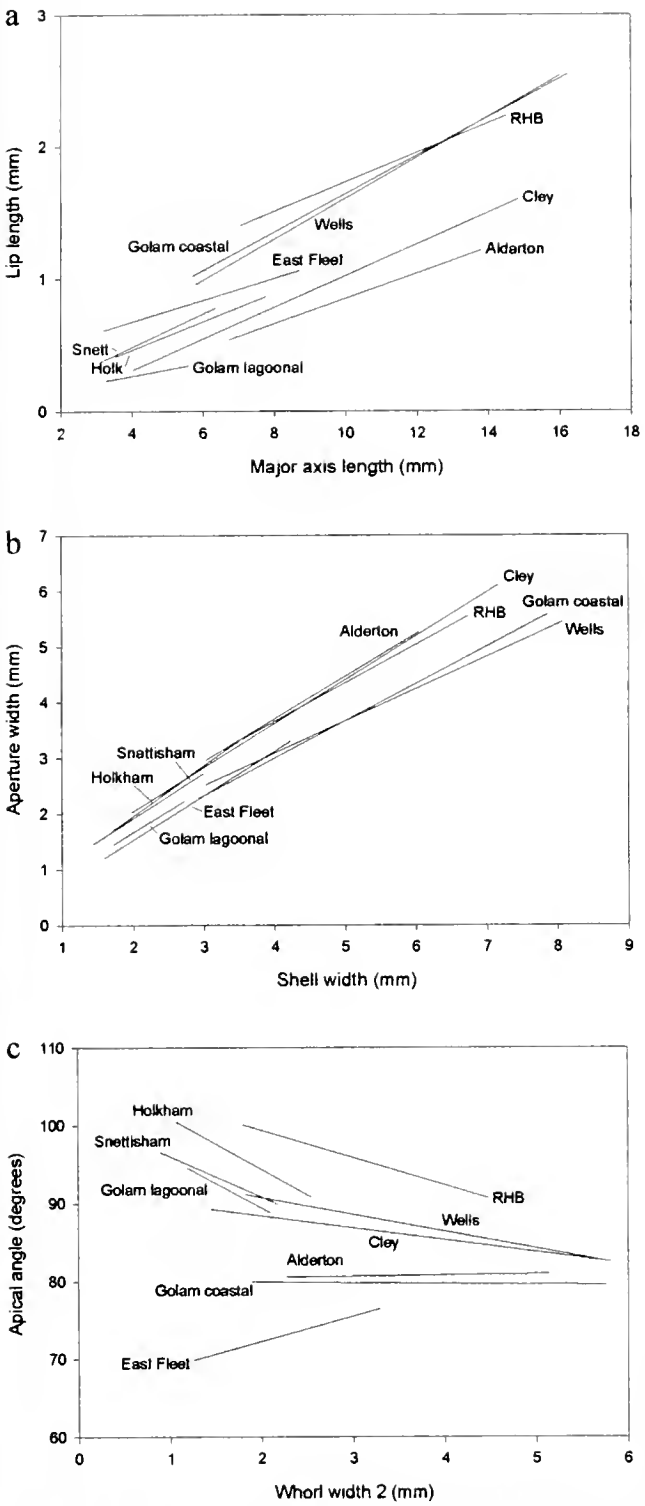

Figure 5. (a) Lip length (mm) plotted against major axis length (mm) with regression lines for each of the nine populations; Holk, Holkham; RHB, Robin Hood's Bay; Snett, Snettisham. (b) Aperture width (mm) plotted against shell width (mm) with regression lines for each of the nine populations; RHB, Robin Hood's Bay. (c) Apical angle (degrees) plotted against whorl width 2 (mm) with regression lines for each of the nine populations; RHB, Robin Hood=s Bay.

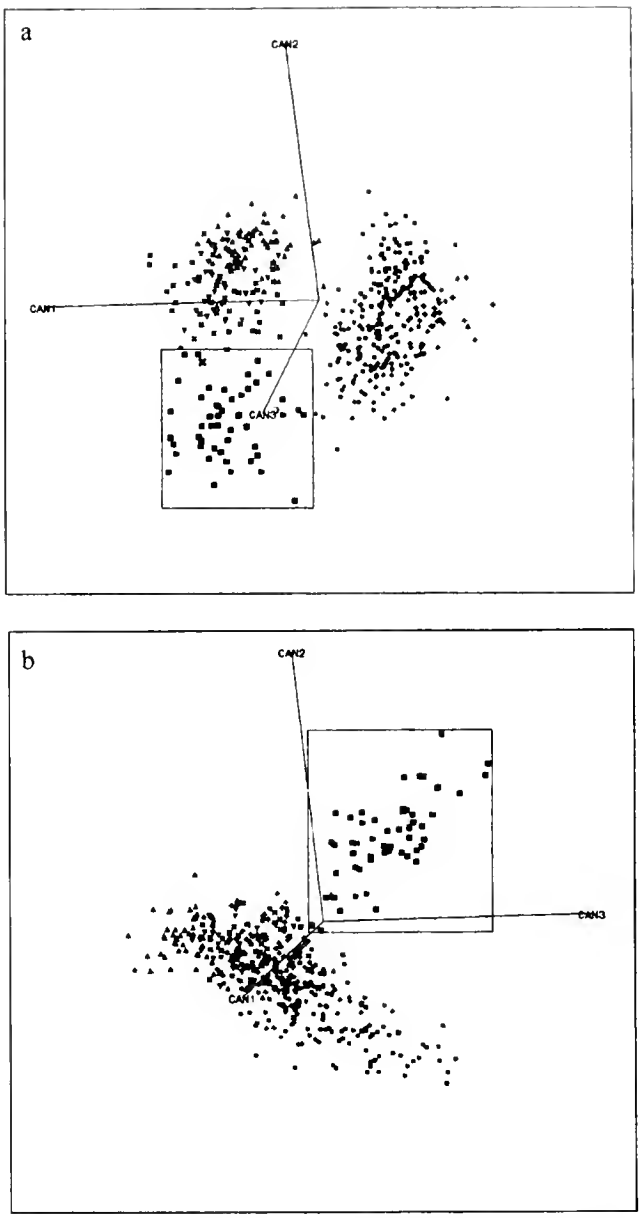

Figure 6. Plot of the data from the nine sites on Canonical Variables 1 (CAN1), 2 (CAN2) and 3 (CAN3). Canonical Variable axes at the mid point. ┼, Alderton; *, Cley; ▲, East Fleet; ▼, Golam coastal; ♦, Holkham; ■, Robin Hood's Bay and Golam lagoonal; ●, Snettisham; ✖, Wells. (a) The shells on the left are the coastal + East Fleet, and are separated from the lagoonal shells along the CAN1 axis. The square encloses the 50 Robin Hood's Bay shells plus one from Wells. (b) The square encloses the 54 lagoonal Golam shells plus one from Alderton.

classifying to Holkham, 9% to Cley and 3% to each of Alderton and East Fleet. Amongst the coastal samples, the highest proportion of misclassifications were from the coastal Golam shells, with 24% misclassifying to Wells and 11% to East Fleet. Twelve percent of Wells shells also misclassified to East Fleet. Of the East Fleet shells, 11% misclassified to coastal Golam and 2% to Cley. In most cases, using the raw data (i.e. not excluding size) reduced the proportion of misclassifications.

DISCUSSION

The *Littorina saxatilis* complex includes a wide variety of shell morphs and the taxon is thought to be undergoing differentiation

TABLE 3.

Discriminant analysis of the data after removing the effects of size by standardizing using the geometric mean.

From	Alderton	Cley	East Fleet	Golam (lagoon)	Golam (coastal)	Holkham	RHB	Snettisham	Wells	Error%	N
Alderton	89	9	0	0	0	0	0	2	0	11	53
Cley	20	60	0	0	0	12	0	8	0	40	50
East Fleet	0	2	87	0	11	0	0	0	0	13	53
Golam (lagoon)	0	4	0	94	0	2	0	0	0	6	54
Golam (coastal)	0	0	11	0	66	0	0	0	24	34	38
Holkham	0	7	0	0	0	83	0	10	0	17	72
RHB	0	0	0	0	0	0	98	0	2	2	50
Snettisham	3	9	3	0	0	19	0	66	0	34	32
Wells	0	0	8	0	12	0	0	0	80	20	50
Total											452

Values are percentages; shading indicates self classification.

(Fretter 1980, Ward & Warwick 1980) which might have arrived at, or in the future reach, species status for one or more of the morphs. This process is thought to be aided by direct development (Van Marion 1981; Janson 1982; Grahame & Mill 1989) and hence poor dispersal ability (Ward & Warwick 1980; Janson 1983; Janson & Ward 1984; Faller-Fritsch & Emson 1985). However, *L. saxatilis* is a rapid colonizer of offshore islands (Johannesson & Johannesson 1995). Furthermore, two other, closely related taxa, *L. arcana* and *L. compressa*, both show comparatively little variation in shell morphology and yet are direct developers. The main reproductive difference between *L. saxatilis* on the one hand and the other two species on the other, is that the former is ovoviviparous whereas the latter are oviparous.

It is clear from the data that the shells in this study can be separated into coastal + the Fleet and lagoonal, and CAN1 provides an axis for this separation. Lagoonal animals have a lighter shell than correspondingly sized coastal animals; also lagoonal shells lack the jugosity found in coastal populations. The position of the Fleet animals is not surprising as this lagoon is tidal and the sample was taken within a few hundred meters of the lagoon entrance. Of the other lagoonal animals, those from Golam (lagoonal) and Holkham satisfy the strict criteria of Muus (1967) for *Littorina tenebrosa*. However, although the shells from Golam (lagoonal) are clearly separable from those of other lagoonal samples, those from Holkham are not. This is somewhat surprising in view of the subjective impression of the shells (Fig. 3). However, this may be due to the (apparent) intermediate shape of the shells from Snettisham between those from Alderton and Cley on the one hand and those from Holkham on the other.

Barnes (1993) has concluded that there is currently insufficient evidence to accept a species status for *L. tenebrosa* (*sensu* Muus 1967) but that it is clearly distinguishable both in shell characteristics and habitat from *L. saxatilis s.s.* He suggested the varietal name *L. saxatilis* var. *lagunae* for the former.

Although, in the current study, the shells from Golam (lagoonal) separate out from those that came from other lagoons, there appears to be a gradation in shape, size and habitat in the lagoonal populations. It would not be surprising if we are witnessing different degrees of divergence in different lagoonal populations. This might be related to the age of the lagoon and hence to the period of their separation from coastal animals. Only those from Golam and Holkham fulfil all of the criteria for the status of *L. saxatilis* var. *lagunae* but others may have changed partially along this route, particularly those from Snettisham. At Snettisham the animals were found apparently permanently submerged but occurred on the substrate (rocks) rather than on macrophytes. At Cley they were on both the substrate and on floating mats of *Chaetomorpha*, and at Alderton were found on the substrate around the edge of the lagoon. Furthermore, in the last two sites, the animals were not permanently submerged and they reached a size similar to that achieved by the coastal animals. The gradation is reflected in the Canonical Discriminant Analysis, where the two taxa do not separate on the CAN2 axis; indeed the Holkham and Golam (lagoonal) shells fall into different groups when aperture width is plotted against shell width. Similarly, they do not separate on the CAN3 axis. Thus, when apical angle is plotted against whorl width 2, the Snettisham shells align with those from Holkham and Golam (lagoonal), but those from Cley and Alderton are more similar to the Wells and coastal Golam shells respectively.

The current view of speciation is generally that of Mayr (1942) in which a geographical barrier develops between populations, isolating them reproductively from each other. Following this, divergence occurs between the populations, even if the separated habitats are identical, and separate species ultimately evolve. However, there is another possible route for speciation, ecological speciation. Although the idea is not new, it has been brought into focus recently that ecological barriers rather than geographical ones may also be important for speciation (Morell 1999). Thus, ecological pressures could favor changes that eventually cause populations to become reproductively isolated in the absence of geographical barriers. It may be expected that populations that are ecologically separated but genetically similar to each other would be more likely to interbreed than comparable ones that have been separated geographically. However, this is not necessarily the case and size differences between genetically similar populations may be sufficient to produce reproductive isolation (Morell 1999).

It is entirely possible that this is the case with *L saxatilis* var. *lagunae* and *L. saxatilis s.s.*, since the former has clearly developed sexual maturity at a size much smaller than occurs in the latter. Indeed Barnes (1993) has suggested that *L. saxatilis* var.

lagunae may have a paedomorphic origin, as Raffaelli (1979) suggested for another taxon within the *L. saxatilis* complex, i.e. *L. neglecta*, and that, in the case of the former, small size is a requirement of living on submerged macrophytes such as *Chaetomorpha*. It follows from the above that *L. saxatilis s.s.* should interbreed with North American *L. saxatilis* but not with *L. saxatilis* var. *lagunae* occurring in the same lagoon. However, caution is required until the appropriate breeding experiments have been attempted. Furthermore, if, as seems to be the case, parallel evolution is occurring in two or more lagoons, and if the individuals in these populations can interbreed with each other but not with adjacent *L. saxatilis s.s.*, then it follows that any resulting "species" will have a polyphyletic origin.

In only one lagoon (Golam) were both *L. saxatilis* var. *lagunae* and *L. saxatilis s.s.* recorded and they were separated by some 100 m, the former occurring at the landward end of the lagoon, the latter adjacent to the rocky shore. It seems highly likely that the two populations are isolated reproductively (Wilson et al. 1999).

From a conservation point of view it is irrelevant as to whether or not we are dealing with two separate species or two morphs. It could be argued that, if priorities have to be decided, for example because of costs, it is more important to conserve at the species level. However, maximum biodiversity must be preserved so that evolutionary processes are allowed to continue. Brackish lagoons are a nationally rare habitat and have been accorded a "priority habitat type" under Annex 1 of the EU Habitats and Species Directive (Bamber 1998). It may be that many lagoons are very teneral, lasting only tens, or at best hundreds, of years (Bamber 1998), but others may be sufficiently permanent to allow complete separation of species to occur. Hence, it is vital that these lagoonal habitats be conserved.

ACKNOWLEDGMENTS

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IS LIFE ON A TROPICAL SHORE REALLY SO HARD?: THE ROLE OF ABIOTIC FACTORS IN STRUCTURING A SUPRALITTORAL MOLLUSCAN ASSEMBLAGE

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ABSTRACT Interactions between biotic and abiotic factors are considered to be the principal mechanisms controlling the dynamics of rocky shore communities. Unfortunately, little research has examined how these factors affect community structure of tropical rocky shore assemblages. We examined the effects of wave action and desiccation on a rocky shore molluscan assemblage on the north shore of Jamaica. This assemblage exists entirely above mean high water (MHW), where physical factors were expected to be more important than biological factors. We compared the molluscan assemblage along ten vertical transects exposed to different levels of wave action and desiccation potential. In all, nineteen species of mollusk were observed, thirteen of which occurred on >50% of our transects. We found no differences in species number, individual densities, or the vertical distribution of the species between transects with differing levels of wave action or desiccation potential. Correspondence analysis revealed differences in assemblage structure, but the differences were not associated with wave action or desiccation, suggesting that these physical factors are not operating at the spatial scale studied. However, gastropod mollusks preferentially occupied pit and crevice microhabitats, which are believed to mediate physical stresses. The distribution of these rocky shore mollusks may be the result of the availability of, and competition for, these sheltered microhabitats.

KEY WORDS: Community structure, rocky shore, mollusks, Littorinidae, *Cenchritis*, *Nodilittorina*, *Tectarius*

INTRODUCTION

The supralittoral fringe, the region on the shore above mean high water (MHW), is often characterized as an especially harsh environment in which species are regulated by physical, instead of biological, factors (Underwood 1979, Lang et al. 1998). High temperatures, desiccation, salinity extremes, UV exposure and mechanical wave stress are all potential abiotic factors that can structure the supralittoral community (Garrity 1984, Lubchenco et al. 1984). These physical factors are considered more extreme along tropical than temperate shores (Moore 1972), and may be a stronger regulating mechanism on these tropical communities (Menge et al. 1986, Menge and Sutherland 1987). Unfortunately, few studies have examined community-level dynamics of the supralittoral fringe habitat in the tropics.

The supralittoral fringe in the Caribbean supports a diverse and conspicuous molluscan assemblage. These species exist in overlapping vertical zones, beginning at or near MHW, and experience varying degrees of wave action, but are seldom, if ever, immersed. Desiccation, heat stress, and mechanical wave action have all been shown to affect the behavior of supralittoral gastropods, resulting in limited cyclic activity (Garrity 1984), selective use of microhabitats (Garrity 1984, Peckol et al. 1989), evaporative cooling (Vermeij 1971), formation of multilayer aggregations (Garrity and Levings 1984), and the establishment of shore-level size gradients (Vermeij 1972). How these physical factors affect the structure of the entire molluscan assemblage, however, is poorly understood.

This paper examines the effects of wave action and desiccation on the distribution of molluscan species on an exposed rocky shore on the north shore of Jamaica. Although several species of this assemblage have been the focus of previous research (Britton 1992, Lang et al. 1998, Gochfeld and Minton 2001), no work has examined community-level dynamics of the entire assemblage.

MATERIALS AND METHODS

The exposed rocky shore near the Hofstra University Marine Laboratory, Priory, St. Ann, Jamaica, is a micro-karsted limestone platform rising approximately three meters above MHW and extending landward from 10 to 25 m. It is often exposed to high swell, but is seldom inundated, remaining, for the most part, dry throughout the year.

We selected and permanently marked ten transects (Fig. 1). Transects ran from MHW landward to the position of first vegetation (usually *Rachicallis americana* (Kuntze, 1891), *Coccoloba uvifera* (Linnaeus, 1758), or *Borrichia arborescens* (Linnaeus, 1758)). MHW was easily identified as the lower boundary of a yellow band of microscopic algae (Brattström 1980). Transects varied in length from 190 to 570 cm, were spaced at least 2 m apart, and extended over approximately 100 m of shoreline. Studies on movement of supralittoral gastropods (Gendron 1977, Peckol et al. 1989, Williams 1995, Williams and Morritt 1995, Gochfeld and Minton 2001) suggest that our transects were sufficiently far apart that individual snails would be unlikely to move between them.

We determined the level of wave energy at each transect line by counting wave impacts over five minute intervals on four different days. If a wave impacted above the MHW, it was counted as a strike. If a wave or its resulting splash landed above the yellow (splash) zone on the rock, it was counted as a severe strike. We conducted all ten surveys on a given day within 30 minutes of each other by randomly dividing the transects between two observers. A preliminary investigation showed no significant bias between the observers. Based on the average number of wave strikes, we divided the transects lines into high energy and low energy sites. We compared the number of strikes for the two groups using a one-tailed, two-sample *t*-test with different sample variances.

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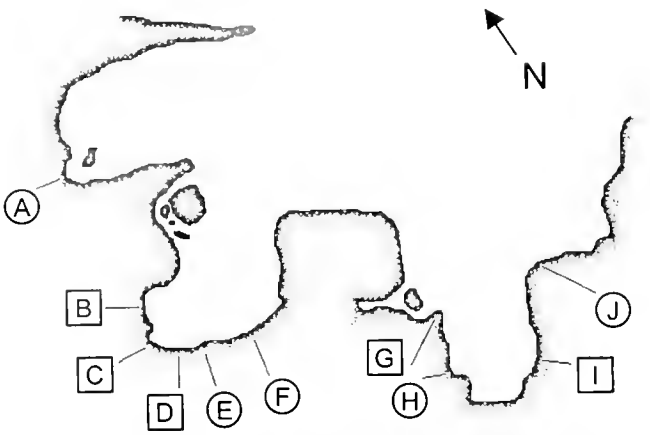

Figure 1. Locations of 10 permanent transects on the exposed shore near the Hofstra University Marine Laboratory, Priory, St. Ann, Jamaica. Squared letters are high energy transects; circled letters are low energy transects.

Desiccation potential on each transect line was measured as the amount of water evaporated from wet sponges after one hour on the shore. Two pre-weighed, 15 × 15 cm sponges, were placed on each transect and wetted with 20 g of water. One sponge was placed at the uppermost end of the transect; the second was placed at the point corresponding to the mid-point of the splash zone. Where necessary, sponges were held in place by monofilament line tied to the rock. After one hour, sponges were placed in labeled re-sealable plastic bags, stored in a cooler and returned to the lab where they were re-weighed using an electronic balance. Prior to use in the field, sponges were dried at 60°C for at least 12 hours. Because we expected wave action to have an effect on desiccation potential, the experiment was repeated on seven calm and seven rough days. Data were analyzed using a three-way ANOVA with transect type (high and low wave energy), vertical shore height (splash zone and above splash zone), and sea condition (calm and rough) as factors.

The slope of the substratum was determined at 15 cm intervals along each transect using a hand-made inclinometer consisting of a t-square, carpenter's level, and pivoting protractor. The device performed adequately, and we were able to reproduce transect profiles on graph paper and estimate the vertical height above MHW at any point along the transect line. Additionally, air and rock temperatures at the uppermost end of each transect line were made using a hand-held thermometer. Rock temperatures were measured by placing the thermometer bulb directly on the rock surface.

To estimate the percent cover of the available microhabitats on our transect lines, we divided a 25 × 25 cm quadrat into a grid with 16 points using string and used the point intercept method to estimate the percent cover of three microhabitat types: (1) exposed surfaces; (2) pits, defined as depressions in the rock large enough to hold a single adult snail of approximately 1 cm length; and (3) crevices, defined as indentations in the rock of sufficient size to hold multiple adult individuals. Quadrants were placed at every meter along each line, and the microhabitat beneath each of the 16 points was determined.

All mollusks within 0.5 m on either side of each transect line were identified to species and their position along the transect line recorded. Several small (<3 mm) individuals of striped littorinids (genus *Nodilittorina*) were found on the shore, and because of difficulties identifying these individuals to species, we designated them as *Nodilittorina* spp. For all coiled gastropods (i.e. littorinids, neritids, etc.), we also recorded the type of microhabitat which they occupied. For each transect line, we determined the position of the splash zone. Using the graphical transect profiles, we converted the positions on the transect lines to vertical heights above MHW. Transects were surveyed over five consecutive days in January 1998 with one randomly selected high and low energy transect surveyed each day.

We tested for differences in species densities, vertical position on the shore, and the vertical breadth of a species' distribution (i.e. range) using MANOVA. Because of limited replication, we reduced the number of dependent variables in the analysis and examined only the five species that occurred on the greatest number of transect lines. Significant results were followed up with multiple univariate ANOVA.

Community structure was compared between transects using correspondence analysis on the species count data, as recommended by Jackson (1997). Only species comprising at least 1% of the total assemblage were included in the analysis.

Data for microhabitat use by transect type was obtained by pooling individuals from the five high and five low energy transects and computing the percent of snails occupying each of the three microhabitats. We examined snail microhabitat use data for differences between high and low energy sites for each of the three microhabitats using *t*-tests.

TABLE 1.

ANOVA on change in sponge weight after 1 hour of exposure on the rocky shore.

Factor	df	SS	F	P
Transect Type (TT)	1	151.6	8.73	0.003
Vertical Height (VH)	1	8416.7	484.7	<0.001
Sea Condition (SC)	1	1299.1	74.81	<0.001
TT × VH	1	176.6	10.17	0.002
TT × SC	1	21.4	1.23	0.268
VH × SC	1	1164.8	67.08	<0.001
TT × VH × SC	1	13.2	0.76	0.384
Error	231	4011.3		
Total	238			

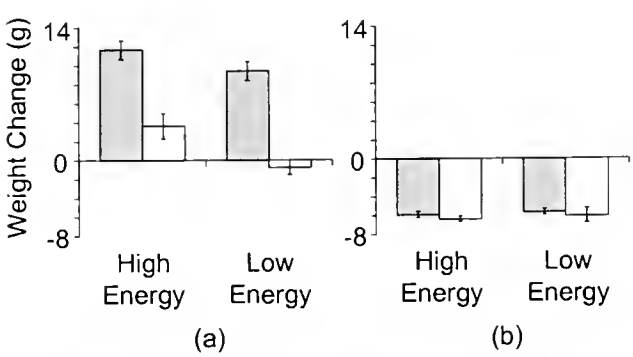

Figure 2. Mean (±SE) change in weight of sponges on high and low energy transects. Sponges were placed at two vertical heights on the shore: (a) in the splash zone; (b) above the splash zone. Solid bars represent replicates conducted on rough days (n = 7). Open bars represent replicates conducted on calm days (n = 7).

RESULTS

High energy transects experienced a significantly greater number of wave strikes (47.9 ± 21.4) compared to low energy transect lines (11.5 ± 8.59; one-tail, two-sample *t*-test, $t = 7.06$, $p < 0.0001$, d.f. = 24). We observed considerable variation in the number of wave strikes from day to day, but regardless of the ocean conditions on any given day, the five high energy transects always experienced greater wave exposure than the low energy transects.

High energy transects also experienced a significantly greater number of severe wave strikes (2.35 ± 3.88) compared to low energy transects (0.5 ± 0.827; one-tail, two sample *t*-test, $t = 2.08$, $p = 0.025$, d.f. = 20). Day to day variation in the number of severe wave strikes was high; on one day, we recorded no severe wave strikes at any of our transects lines, but on another day, we recorded as many as 15 severe wave strikes in a five minute interval at a high energy transect compared to a maximum of one strike at our low energy transects.

Desiccation potential depended upon transect type (high energy or low energy), shore height (in or above the splash zone), and sea conditions (rough or calm) (Table 1). Sponges placed above the splash zone lost on average 5.92 ± 1.62 g of water, regardless of transect type or surf conditions. Change in weight for sponges placed in the splash zone varied with surf conditions and transect type. On rough days, sponges in the splash zone gained an average of 10.49 ± 5.5 g of water regardless of transect type (Fig. 2). On calm days, no significant change in sponge weight was observed (1.45 ± 6.27 g), but sponges at high energy transects often gained water while sponges at low energy transects usually lost water. This result, however, was not manifested in a significant three-way interaction in the ANOVA (Table 1).

Nineteen species of mollusk were recorded during this study (Table 2). All were gastropods, except for two species of polypla-

TABLE 2.

Mean height on the shore (±sd) and density (±sd) for all mollusk species on a Jamaican shore.

Species	Transects occupied		Mean height on shore (cm)	Mean density (Indiv./m^2)	Total individuals
Nodilittorina dilatata (d'Orbigny, 1842)	high	5	69.2 (11.6)	0.311 (0.274)	495
	low	5	73.7 (9.6)	0.524 (0.758)	641
Cenchritis muricatus (Linnaeus 1758)	high	5	112.5 (19.5)	0.117 (0.033)	207
	low	5	118.8 (8.75)	0.155 (0.058)	230
Nodilittorina angustior (Mörch, 1876)	high	4	72.9 (20.1)	0.176 (0.159)	222
	low	4	81.0 (18.56)	0.157 (0.256)	151
Nodilittorina riisei (Mörch, 1876)	high	3	39.3 (9.3)	0.241 (0.297)	229
	low	3	45.7 (8.7)	0.135 (0.150)	113
Nodilittorina zigzac (Gmelin, 1791)	high	5	55.9 (25.1)	0.046 (0.029)	79
	low	4	68.6 (20.1)	0.047 (0.031)	71
Nerita versicolor (Gmelin, 1791)	high	5	50.5 (20.8)	0.034 (0.024)	64
	low	3	65.8 (16.1)	0.063 (0.037)	61
Nodilittorina spp.	high	3	44.1 (24.7)	0.077 (0.113)	70
	low	2	36.2 (25.4)	0.075 (0.078)	37
Acanthopleura granulata (Gmelin, 1791)	high	5	15.20 (4.7)	0.051 (0.039)	83
	low	4	10.55 (9.1)	0.016 (0.012)	13
Notoacmaea antillarum (Sowerby, 1831)	high	4	17.1 (10.6)	0.033 (0.022)	42
	low	5	14.3 (11.4)	0.033 (0.019)	42
Tectarius antonii (Philippi, 1846)	high	4	85.9 (15.8)	0.017 (0.008)	23
	low	4	82.0 (20.1)	0.050 (0.074)	48
Nerita tessellata (Gmelin, 1791)	high	4	36.2 (22.0)	0.024 (0.029)	40
	low	2	40.7 (41.5)	0.017 (0.019)	7
Chiton tuberculatus (Linnaeus, 1759)	high	3	14.3 (11.2)	0.006 (0.002)	8
	low	3	20.9 (18.9)	0.018 (0.012)	20
Planaxis lineatus (da Costa, 1778)	high	3	40.2 (0.7)	0.013 (0.018)	12
	low	3	48.5 (9.4)	0.006 (0.004)	6
Nodilittorina mespillum (Mühlfield, 1842)	high	1	32	0.031	9
	low	1	76	0.009	5
Nerita peloronta (Linnaeus, 1758)	high	1	88	0.003	1
	low	2	87.5 (24.7)	0.009 (0.008)	5
Fissurella nodosa (Born, 1778)	high	2	12.3 (2.9)	0.002 (0.000)	2
	low	0	—	—	—
Fissurella barbadensis (Gmelin, 1791)	high	0	—	—	—
	low	1	74.5	0.002	1
Diodora cayenensis (Lamarck, 1822)	high	1	14	0.003	1
	low	0	—	—	—
Thais rustica (Lamarck, 1822)	high	1	0	0.004	1
	low	0	—	—	—
Thais sp.	high	1	8.5	0.002	1
	low	0	—	—	—

cophorans. The number of species did not differ significantly between high energy (11.40 ± 1.67) and low energy (9.80 ± 3.63) transects (two sample *t*-test, $t = 0.89$, $p = 0.41$, d.f. = 5). *Nodilittorina dilatata* (d'Orbigny, 1842) and *Cenchritis muricatus* (Linnaeus, 1758) were the most abundant snails at both high and low energy transects, forming >30% of each assemblage. The majority of species present formed less than 5% of the assemblage.

Few differences were found in the species composition between high and low energy transects. Thirteen species occurred on >50% of the transect lines. Three species (*Nodilittorina ziczac* (Gmelin, 1791), *Acanthopleura granulata* (Gmelin, 1791), and *Notoacmaea antillarum* (Sowerby, 1831)) occurred on 90% of the transect lines and two species, *Nodilittorina dilatata* and *C. muricatus*, were present on every transect line. Four species (*Thais rustica* (Lamarck, 1822), *Thais* sp., *Diodora cayenensis* (Lamarck, 1822), and *Fissurella barbadensis* (Gmelin, 1791)) were each represented by a single individual and another, *F. nodosa* (Born, 1778) was represented by two individuals. With the exception of *F. barbadensis*, these rare species were found on high energy transects.

The first two components from the correspondence analysis explained 70.8% of the variation (Table 3). A scatter plot of the components (Fig. 3) showed six of the ten transects clustering near the origin. Transects D, E, and F fell outside the cluster as a result of a low component 1 value. *Cenchritis muricatus* contributed most heavily to component 1 (Table 3) and formed a large proportion of the community on these transects (68.8, 77.1 and 41.7%, respectively). Transect 1 is also distinguishable from the cluster due to a low component 2 value. Component 2 receives a large contribution from *Nodilittorina riisei* (Mörch, 1876), and to a slightly lesser extent from *Nodilittorina* spp. and *N. dilatata* (Table 3). The assemblage at Transect 1 is differentiated from other transects by its relatively low percentage of *N. dilatata* (32.5%) and its high percentage of *N. riisei* (25.1%).

Total mollusk densities did not differ significantly between high energy and low energy transects (Mann-Whitney test; $W = 27$; $p = 1.000$). Densities of the five most common species (*Acanthopleura granulata*, *Cenchritis muricatus*, *Nodilittorina dilatata*, *N. ziczac*, and *Notoacmaea antillarum*) also were not significantly different (MANOVA, $\Lambda = 0.353$, $p = 0.367$, d.f. = 5,4). Unfortunately, we did not have sufficient replication to include all species in our analysis, and some trends could not be tested statistically. Densities of *Nodilittorina riisei* on high energy transects were twice those observed on low energy transects, although variation was high. The opposite trend was noted for *Tectarius antonii* (Philippi, 1846) and *Chiton tuberculatus* (Linnaeus, 1759) (Table 2). Again, variation was high, requiring that these observations be viewed with caution.

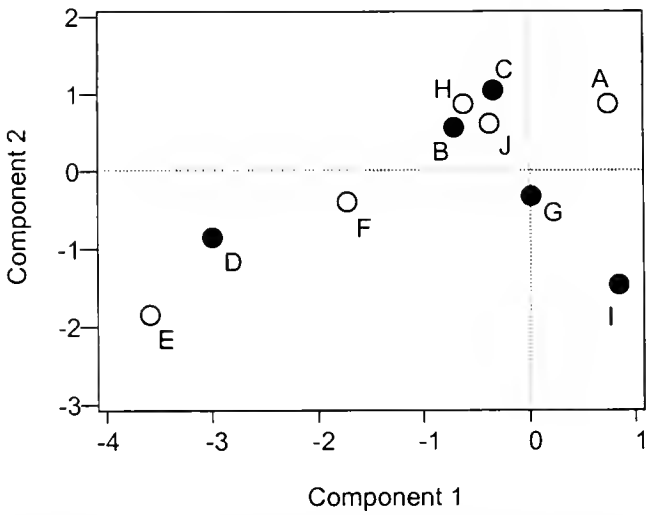

Figure 3. Scatter plot of the scores for components 1 and 2 from the correspondence analysis of the molluscan assemblage. Each point represents one transect line. Open circles are low energy transects; solid circles are high energy transects.

The vertical height on the shore for the five most common species did not differ between high and low wave energy transects (MANOVA, $\Lambda = 0.097$, $p = 0.504$, d.f. = 5,1). No difference between high and low energy transects was observed for the vertical ranges of *A. granulata*, *Nodilittorina dilatata*, *N. ziczac*, and *Notoacmaea antillarum* on the shore (MANOVA, $\Lambda = 0.508$, $p = 0.758$, d.f. = 4,2). We excluded *C. muricatus* from this analysis because its distribution extended beyond the upper limit of our transects.

The five most common species occupied three distinct positions on the shore (ANOVA, $F = 88.68$, $p < 0.001$, d.f. = 4,42; followed by Tukey pairwise comparisons with an overall error of 0.05) (Fig. 4). *Cenchritis muricatus* occurred at the highest shore position, with an average height of 115.67 cm above MHW. *Nodilittorina dilatata* and *N. ziczac* occurred on the mid-shore at average vertical heights of 71.46 and 61.54 cm, respectively. *Acanthopleura granulata* and *Notoacmaea antillarum* occurred lowest on the shore, at 13.13 and 15.55 cm, respectively.

Coiled gastropods preferentially occupied pit microhabitats, regardless of microhabitat availability or vertical position on the shore ($\chi^2 = 428.2$, d.f. = 2, $p < 0.0001$). The exceptions were *C. muricatus*, which showed no preference for pits over crevices and *Nerita versicolor* (Gmelin, 1791), which preferentially occupied crevices at high energy transects (Table 4). Overall, 70.9% of the observed gastropod occupied pits, 23.9% occupied crevices, and only 5.2% occupied exposed surfaces. Conversely, exposed surfaces accounted for >70% of available microhabitats at all levels of the shore (Fig. 5). The availability of microhabitats did not vary between high and low energy transects.

Use of exposed surfaces by gastropods was significantly higher

TABLE 3.

The coordinate (Coor) and contribution (Cont) values for the first two components of the correspondence analysis.

	Component 1		Component 2	
Species	Coor	Cont	Coor	Cont
Nodilittorina dilatata	0.228	0.074	0.198	0.159
Cenchritis muricatus	−1.083	0.636	−0.114	0.020
N. angustior	0.297	0.041	0.130	0.023
N. riisei	0.480	0.098	−0.546	0.366
N. ziczac	−0.259	0.013	0.337	0.061
Nerita versicolor	−0.366	0.021	0.214	0.021
Nodilittorina spp.	0.633	0.053	−0.583	0.130
Acanthopleura granulata	−0.064	0.000	−0.403	0.056
Notoacmaea antillarum	−0.741	0.057	−0.430	0.056
Tectarius antonii	0.198	0.003	0.564	0.081
Nerita tessellata	0.270	0.003	−0.453	0.027
Proportion explained	.5259		.1822	
Cumulative	.5259		.7081	

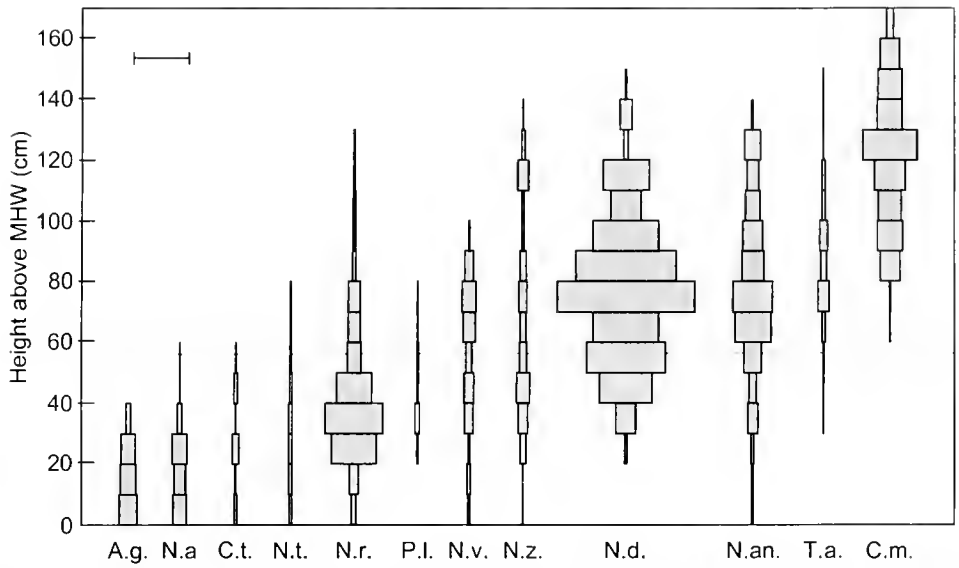

Figure 4. Distribution of the 12 most abundant species of supralittoral mollusks on the exposed shore near the Hofstra University Marine Laboratory, Priory, St. Ann, Jamaica. Dashed line represents the approximate upper limit of the splash zone. Bar in the upper left corner is equivalent to 10 individuals per m^2. A.g. = *Acanthopleura granulata*; N.a. = *Notoacmaea antillarum*; C.t. = *Chiton tuberculatus*; N.t. = *Nerita tessellata*; N.r. = *Nodilittorina riisei*; P.l. = *Planaxis lineatus*; N.v. = *Nerita versicolor*; N.z. = *Nodilittarina ziczac*; N.d. = *Nodilittorina dilatata*; N.an. = *Nodilittorina angustior*; T.a. = *Tectarius antonii*; C.m. = *Cenchritis muricatus*.

on high energy transects than on low energy transects (*t*-test, $t = 2.73$, $d = 8$, $p = 0.022$). No significant differences were observed for pit or crevice use between high and low energy transects.

DISCUSSION

Wave action and desiccation stress have been identified as important abiotic stresses structuring intertidal communities (Garrity 1984, Menge et al. 1986), particularly for rocky shore gastro-

TABLE 4.

Percent of snails occupying pits, crevices, and exposed surfaces.

Species		Pits (%)	Crevices (%)	Exposed surfaces (%)
Cenchritis muricatus	high	49.1	39.5	11.4
	low	44.7	49.4	6.0
Nodilittorina riisei	high	80.4	12.9	6.7
	low	70.6	20.2	9.2
Nodilittorina angustior	high	76.5	15.0	8.5
	low	72.7	24.0	3.3
Nodilittorina ziczac	high	60.5	25.9	13.6
	low	69.0	21.1	9.9
Nodilittorina dilatata	high	73.0	20.3	6.7
	low	75.5	21.9	2.6
Tectarius antonii	high	52.2	21.7	2.6
	low	79.2	12.5	8.3
Nodilittorina spp.†	high	78.9	1.4	19.7
	low	88.2	2.9	8.8
Nerita tessellata	high	62.2	2.7	35.1
	low	57.1	14.3	28.6
Nerita versicolor	high	27.0	54.0	19.0
	low	60.9	31.3	7.8
Planaxis lineatus	high	66.7	25.0	8.3
	low	100.0	0	0

† Includes all *Nodilittorina* individuals <2 mm

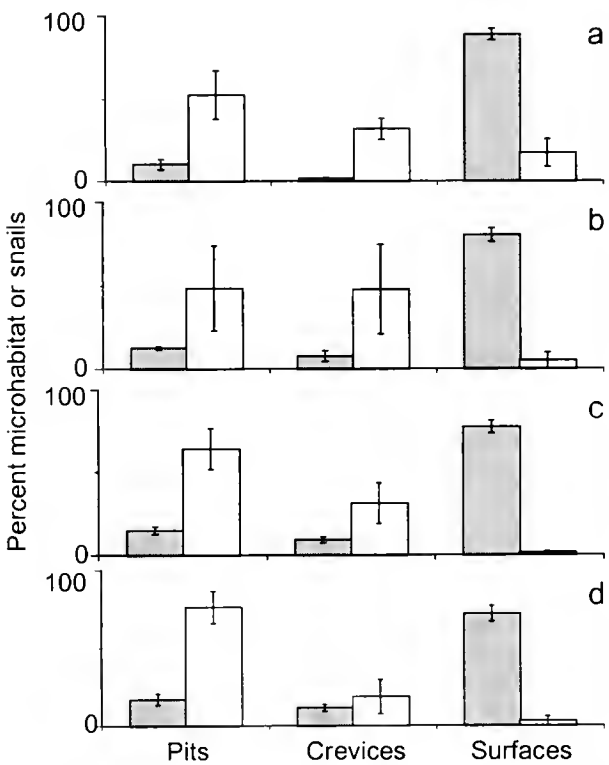

Figure 5. Percent microhabitat availability and use by supralittoral mollusks at four heights above MHW. Data are means (±SE) for 10 transect lines. Solid bars represent the relative abundance of pits, crevices, and exposed surfaces available to snails and open bars represent the percent of snails occupying pits, crevices, and exposed surfaces. (a) 4 m above MHW; (b) 3 m above MHW; (c) 2 m above MHW; (d) 1 m above MHW.

pods occupying the supralittoral fringe. On exposed shorelines, the splash zone directly above MHW is subject to intense mechanical stress from wave action. The ability to adhere to the substrate when under severe impact from waves should be a major factor controlling the distribution of species (Rafaelli and Hughes 1978), as individuals knocked from the substrate are likely to be swept away, crushed, or fall victim to predation (Levings and Garrity 1983, Garrity and Levings 1984).

Many supralittoral gastropod species remain emersed, which may reduce predation from subtidal predators (Levings and Garrity 1983, Garrity and Levings 1984). Emersion also increases their risk of desiccation and heat coma from exposure to high temperatures. Temperatures on exposed rocky shores in the tropics are often sufficient to cause mortality in supralittoral gastropods (Garrity 1984, Garrity and Levings 1984). Laboratory studies on the heat coma temperatures (HCT) of five species of Caribbean littorinids from Jamaica, indicate that daily temperatures at the study site can exceed the lethal limits for these species (Britton 1992). During this study, ambient rock temperatures of 45°C exceeded the lethal levels for two of the five littorinid species.

Our results suggest that neither wave action nor desiccation are important factors structuring the molluscan assemblage at this location. Species numbers, densities, assemblage composition, and the range of distribution of individual species were constant across the physical factors investigated. Correspondence analysis revealed that four transects were different from the others (Fig. 2), but three of these transects (D, E, and F) were related more by geographical proximity to one another than by transect type, and this suggests that relatively small scale, localized events are acting on the assemblage.

Other studies have correlated habitat heterogeneity with the distribution of rocky shore mollusks (Peckol et al. 1989), and we observed a significant preference among the supralittoral gastropods for the less abundant pit and crevice microhabitats over exposed surfaces. Because our transects did not vary in microhabitat availability, the absence of differences in the molluscan assemblage between high and low energy transects could be explained if microhabitat availability is the primary factor structuring the community. Unfortunately, microhabitat availability alone is not an adequate mechanism: microhabitats should act indirectly on community structure by altering mortality levels caused by physical or biological mechanisms.

Pit and crevice microhabitats may be cooler and more humid than exposed surfaces, resulting in lower desiccation stress and ambient temperature (Levings and Garrity 1983, Garrity 1984). These microhabitats may also provide protection from mechanical stress (Raffaelli and Hughes 1978), reducing the likelihood of individuals being swept from the substrate when struck by waves.

Patterns of microhabitat use between high energy and low energy transects suggest that pits and crevices may be important in alleviating desiccation stress. On high energy transects, nearly all species were found more frequently on exposed surfaces (Table 3). If pits and crevices were important in reducing the risk of being swept from the shore by wave action, we would expect fewer snails on exposed surfaces at high-energy sites. As this was not the case, we believe wave action to have an insignificant direct effect on the distributions of these species. Indirectly, high wave action may lower desiccation potential and rock temperature compared to low energy sites and therefore, individuals on exposed surfaces would be less likely to suffer mortality from desiccation along high energy transects.

In contrast to our findings, Lang et al. (1998) found little preference for pits and crevices over exposed surfaces for *Nodilittorina angustior*, *N. riisei*, and *N. antonii* in research conducted on nearly the same area of the Jamaican shoreline. However, rock temperatures during this earlier study were approximately 6°C lower than during our study and were well below the measured HCT for these species; this may have allowed the species to distribute themselves in a more random manner over the rock surface.

McMahon (1990) has suggested that mollusks occupying the supralittoral fringe have sufficient physiological tolerances to high temperature and desiccation that these factors play little role in determining zonation patterns. Britton (1992) found only a weak relationship between desiccation resistance and HCT and the relative positions on the shore for seven species of Caribbean littorinids. The littorinid species examined possessed high desiccation resistance and were able to survive from 12 to 30 days of emersion (Britton 1992). Interestingly, the lowest HCT was for *C. muricatus* (42.1°C), the littorinid occupying the highest position on the shore.

Unfortunately, methods used to determine HCT temperatures entail submerging the animal in a heated water bath. In nature, these species are seldom, if ever, submerged. This casts doubt on the ecological relevance of this type of study (Garrity 1984). Mortality of supralittoral gastropods in the tropics has been linked to elevated temperatures (Levings and Garrity 1983), and even if desiccation and temperature are not directly responsible for mortality, they may indirectly lead to mortality via some other factor (Menge and Sutherland 1987). Therefore, any means of ameliorating temperature stress would be advantageous to the individual.

We do agree, however, with McMahon's (1990) contention that the distributional patterns observed for tropical rocky shore mollusks may be the result of a suite of factors, including the physical, biological, and physiological environment. Because pits and crevices account for <30% of the available habitat, competition for these microhabitats may be an important factor controlling species distributions and ultimately, community structure. The interaction of biological (i.e. competition and predation) and physical (i.e. desiccation and temperature stress) factors may be responsible for the distribution of supralittoral gastropods on exposed tropical shorelines.

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EFFECTS OF TEMPERATURE AND DESICCATION ON TISSUE URIC ACID DYNAMICS IN *LITTORINA SAXATILIS* (OLIVI)

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ABSTRACT Previous studies have shown (Smith & Smith 1998) that the tissue concentration of uric acid in *Littorina saxatilis* varies considerably on a seasonal basis. In order to investigate possible physical parameters that might account for this variation, periwinkles were kept in controlled temperature rooms at 25°C or 10°C, and either immersed in seawater or dry (emersed). The combination of 25°C and emersion caused the highest concentration of uric acid, and 10°C and immersion the lowest. Returning emersed snails to seawater caused the tissue uric acid concentration to decline. This re-immersion also produced an increase in ammonia excretion. It appears that uric acid accumulates during emersion, which would mitigate water loss, and that this uric acid is re-converted to ammonia for excretion during subsequent immersion. The time course for these changes is such as to accommodate the natural cycle of immersion/emersion.

KEY WORDS: *Littorina saxatilis*, uric acid, ammonia, temperature, desiccation

INTRODUCTION

Research on the influence of environmental factors on uric acid concentration in intertidal gastropods began with the classic papers by Needham (1935, 1938) with substantial contributions later by Potts (1967). These workers demonstrated that species located high in the intertidal zone contain a relatively greater amount of uric acid than those from lower. Previous reports indicate (Smith et al.1995) that shore location results in differences in uric acid levels within species (*Littorina saxatilis* and *L. arcana*), with crevice-dwelling animals having a lower uric acid concentration than animals collected from the surface of boulders.

The principal end product of nitrogen metabolism has long been recognized to be related to the amount of water that is available for its excretion. Aquatic animals are able to excrete toxic ammonia since water is plentiful and can readily flush ammonia from the body. Terrestrial species of gastropods (and other animals), however, are ureotelic or uricotelic (Delauney, 1931, Needham, 1935), since urea and uric acid are relatively non-toxic and can therefore be permitted to accumulate and ultimately be excreted with a minimal volume of water. The disadvantage of urea and uric acid is that they are metabolically more costly to synthesize than ammonia. Uric acid is the most costly, but is also the most conservative of water. Since it is highly insoluble, it may be stored in tissues without detrimental osmotic effect, and may, in those animals that excrete uric acid, be excreted in crystalline form with very little water loss. Whether in fact periwinkles actually excrete uric acid as such is questionable; Heil and Eichelberg (1983) reported that the only excreted metabolite they were able to detect in *L. littorea* was ammonia.

Littoral gastropods may be thought of as being intermediate between aquatic and terrestrial snails since they are subjected to alternating periods of immersion with plentiful seawater, and of emersion with the possibility of dehydration. Under such conditions it is to be expected that seasonal changes would have a significant effect on the accumulation of uric acid since the presumed factors that stimulate uric acid accumulation, namely increased temperature and desiccation, will be increased in summer and reduced in winter. This has been shown in land snails (Jezewska et al. 1963; Laziradou-Dimitriadou & Kaloyianni, 1989), and, in an earlier paper (Smith & Smith 1998) we were able to show that there is indeed a much greater concentration of uric acid in winkles in summer than in winter. Although it seems reasonable to assume that the physical parameters that lead to accumulation of uric acid in the summer are temperature and desiccation, it is necessary to test the effects of these factors under controlled laboratory conditions. For this reason, winkles were brought to the laboratory to be maintained in controlled conditions of temperature and desiccation in order to independently test these factors on the animal's uric acid content. Further, the ultimate fate of this uric acid, conversion to ammonia, was investigated.

MATERIALS AND METHODS

Collections

Samples of *Littorina saxatilis* were collected from a boulder field at Filey Brigg, North Yorkshire, on the northeast coast of England (British National Grid Reference TA 133814; this location is labeled "B" in Smith et al. 1995). All animals used in this study were collected between February 10 and April 19,1999.

Treatments

The animals were transported to the laboratory where they were maintained in temperature controlled rooms either at 10° (designated here as "C") or 25° (designated as "H"). In each of these rooms animals were separated into either a dry aquarium ("D") or an aquarium where they were kept submerged in seawater ("W") by means of a nylon net attached to the walls of the aquaria at the surface of the water. With each collection of winkles, a number of algae-covered stones were taken from the same shore and were placed on the bottom of the aquaria (Warwick 1983). This made it possible to keep the winkles in good condition well beyond the duration of the experiment. The winkles were maintained in these conditions for at least 48 hours before being assayed for uric acid.

In order to determine whether and how much animals that had been adapted to dry conditions would reduce their uric acid con-

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centration upon re-immersion, a sample of 45 C/D animals was transferred to C/W conditions. An equal sized sample of H/D was transferred to H/W and forty-eight hours after transfer, each sample was assayed for uric acid.

Most determinations were done after 48 hours in the chosen temperature/moisture conditions. To determine whether the rate of uric acid accumulation during desiccation would occur within a time frame that corresponds to typical emersion cycles in nature, winkles were placed in H/D and then uric acid concentration in samples of 15 animals immediately measured, at 3 hours, 6 hours, 18 hours, 28 hours, and 48 hours.

Uric Acid Determination

This method is the same as that reported in earlier papers (Smith *et al* 1995, Smith & Smith 1998). The shells were cracked and the body was removed under a dissecting microscope. The body was weighed and then homogenized in 100 µl of phosphate buffer (pH 7.8) at room temperature. After centrifugation the supernatant was removed and frozen for later analysis for uric acid.

The supernatant was assayed using a modification of the Sigma Diagnostics procedure no. 685. This procedure essentially follows the method of Duerr (1967) and utilizes uricase to promote the oxidation of uric acid to allantoin, CO_2 and H_2O_2. The H_2O_2 then reacts, in the presence of peroxidase, with 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenesulphonate to form a quinoneimine dye. The intensity of the color formed is proportional to the concentration of uric acid and is read at 520 nm. Concentration is reported as µg of uric acid per gram of wet tissue weight (µg g^{-1}).

Body Weight

The wet weight of four samples of 20 winkles was obtained by weighing each animal individually after blotting external water with filter paper. Each group of snails was then placed in one of the four-temperature/ moisture conditions for 5 days, at which time the animals were individually weighed once more. This was to determine possible changes in body weight, especially in those animals that had been subjected to desiccating conditions.

Ammonia Production

Forty-five animals were subjected to H/D for 48 hours; another 45 animals were placed in H/W for the same period of time. They were then placed, in groups of 15 animals, in 30 ml of seawater at 25°C. Their rate of ammonia production was determined by measuring the concentration of ammonia in the seawater over a 4 h period using an Omega ammonia colorimeter. The rate of production was expressed as µM NH_3 g^{-1} h^{-1}.

Statistics

Non-parametric methods were used for all statistics reported in this paper: two-way analysis of variance using the method of Tukey, comparison of individual means by the Mann-Whitney rank sum test, and paired tests by the Wilcoxan method.

RESULTS

Temperature and Desiccation Effects

The effects of the four combinations of temperature and moisture treatments: 10°C and immersed (C/W), 25°C and immersed (H/W), 10°C and dry (C/D), 25°C and dry (H/D) are illustrated in Figure 1. The bar labeled "pre" is the mean uric acid concentration

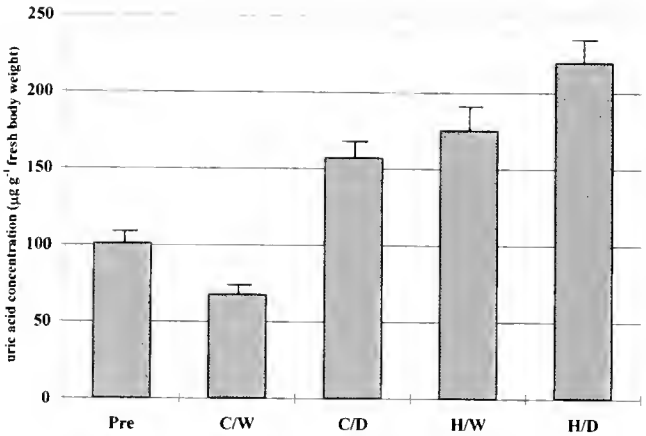

Figure 1. Mean (± SEM) wet tissue uric acid concentration (µg g^{-1}) in Littorina saxatilis maintained at the indicated conditions of temperature and moisture for 48 h. Pre = control fresh from the shore (N = 42); C/W = 10°C immersed (N = 39); C/D = 10°C emersed (N = 45); H/W = 25°C immersed (N = 38); H/D = 25°C emersed (N = 36).

of 42 periwinkles measured immediately upon arrival at the laboratory. The fact that this is higher than the next bar, which is the mean of 39 animals 48 hours after immersion in seawater at 10°C, is probably because the animals had already been emersed for some hours by the time they arrived at the laboratory, since the collections were taken at about the time of low water. The C/W winkles showed the lowest concentration of uric acid, and the H/D animals the highest.

Table 1 shows the results of statistical testing of the data by the Tukey non-parametric method. Clearly, both temperature and desiccation have highly significant effects on uric acid concentration, but there is no interaction between the two factors. Testing between individual means (Mann-Whitney) reveals highly significant differences ($P < 0.001$) when comparing C/W vs C/D, H/W vs C/W, and C/W vs H/D; significant differences ($P = 0.022$) with H/D vs H/W and H/D vs C/D; and no significant difference between H/W and C/D.

Effect of Re-hydration

It was clear that desiccation leads to an increase in uric acid concentration. What if the animals are now re-immersed in seawater? Samples of winkles ($n = 45$) were kept in H/D and C/D conditions for 48 hours to raise their uric acid concentration. After 48 hours half of the surviving animals in each sample were assayed for uric acid and the remaining animals in each sample were immersed in seawater at the same temperature and assayed for uric acid 48 hours later. The results are shown in Figure 2. ANOVA of the data of Figure 2 is highly significant ($P < 0.001$). Testing of

TABLE 1.

Anova table of the effects of temperature and desiccation on uric acid concentration in *Littorina saxatilis*.

Source of variation	*MS*	*F*	*P*
Temperature	284284	46.2	<0.001*
Desiccation	177169	28.8	<0.001*
Interaction	20116	3.3	n.s.

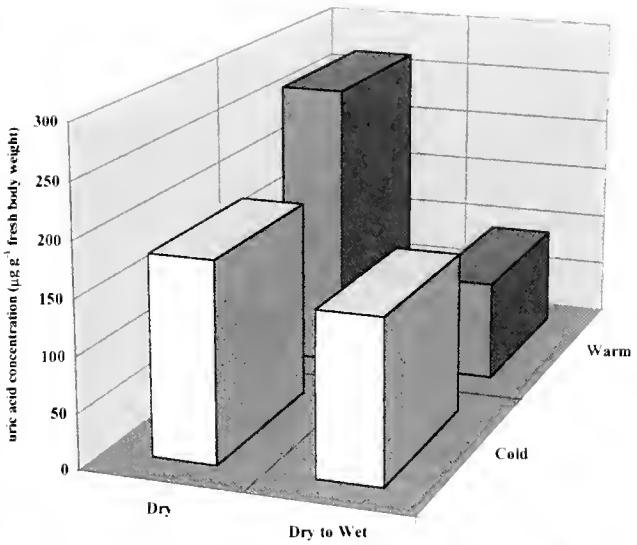

Figure 2. Effect of transfer of Littorina saxatilis after 48 h desiccation ("dry", either warm [25 C] or cold [10 C]) to 48 h immersion in seawater ("dry to wet", either warm or cold) on mean wet tissue uric acid concentration (μg g^{-1}). N = 19 (C/D), 24 (C/D to C/W), 18 (H/D), 15 (H/D to H/W).

individual sample responses to re-hydration shows a highly significant ($P < 0.001$) reduction in uric acid when winkles are moved from H/D to H/W, but the response to a move from C/D to C/W is considerably less ($P = 0.17$); not statistically significant at $\alpha = 0.05$.

Weight Loss during Desiccation

Upon desiccation, animals will almost certainly have some reduction in weight as a result of water loss. To determine whether such loss of weight is sufficient to account for the concomitant increase in uric acid concentration, four groups of 45 snails were initially weighed, then each group put into one of the four temperature/moisture conditions for 5 days. Table 2 shows that the animals that had been kept submerged had a very slight (not significant) gain in body weight. Desiccated animals had a significant ($P < 0.001$) weight loss. However, if the % change in weight is compared with the % change in uric acid as a result of desiccation (data from Fig. 1) it is clear that the loss of body weight cannot account for the large increase in uric acid concentration. Animals kept C/D had a 6.2% weight loss, but a 43.0% increase in uric acid concentration; those animals maintained H/D had a 6.0% weight loss but a 79.8% gain in uric acid.

Rate of Increase in Uric Acid during Desiccation

The determinations described in the preceding sections were done following 48 h of exposure to the chosen temperature/moisture conditions. Winkles were placed in H/D and then samples of 15 snails were immediately assayed, at 3 h, 6 h, 18 h, 28 h, and 48 h. This was done to determine whether the uric acid accumulation that is due to desiccation would occur within the time constraints of a typical immersion/emersion cycle in nature. The results are illustrated in Table 3, and show that there is indeed a significant increase in uric acid by 6 h, within the emersion time of a normal tide cycle for these animals.

Ammonia Production

Forty-five periwinkles were conditioned to H/D for 48 h to increase their uric acid concentration. The control was an equally sized sample held at the same temperature but kept immersed. After 48 h, groups of 15 animals were placed in 30-ml seawater and their ammonia production was measured over a 4-h period. The 45 animals that had been H/D had an ammonia production rate of 0.41 μM NH_3 g^{-1} h^{-1}, while the H/W controls produced at a rate of 0.25 μM NH_3 g^{-1} h^{-1}. These rates are consistent with those reported by Uglow and Williams (2001) for Hong Kong littorines. Statistical testing between these means was not done, since, although 45 animals were in each sample, they were combined into groups of 15, and 3 experimental and 3 control samples are insufficient for statistical testing.

DISCUSSION

Shore location and seasonal effects suggest that the determining physical causes of increased uric acid concentration are probably temperature and desiccation. This hypothesis is borne out by the experiments reported here using temperature-controlled rooms to carefully control the temperature of periwinkles that were either completely submerged in seawater or kept in dry conditions. Both increased temperature and desiccation caused significant gains in uric acid burden of *L. saxatilis*. While no interaction between these two physical factors was demonstrated, one would expect that the effect of temperature would be manifested by an enhancement of the drying effect. However, the actual loss of body weight due to desiccation was in fact rather slight, and was no greater at 25°C than it was at 10°C. Possibly, then, the temperature effect is to metabolically increase the rate of synthesis of uric acid.

Heil and Eichelberg (1983), and Eichelberg and Heil (1989) have suggested that uric acid serves as a temporary storage form of nitrogenous wastes in *Littorina littorea* during times of water lack and low salinity. They were unable to detect any excreted form of nitrogen other than ammonia. If the uric acid were a temporary

TABLE 2.

The effects on whole body weight (g) of 5 days of treatment under various temperature/moisture conditions.

Treatment	*N*	Initial weight (mean ± SEM)	Final weight (mean ± SEM)	Δ Weight	% Δ Weight	*P*
C/W	20	0.407 ± 0.032	0.410 ± 0.031	0.003	1.1	0.381
C/D	20	0.474 ± 0.040	0.451 ± 0.038	−0.28	−6.2	<0.001*
H/W	20	0.387 ± 0.038	0.389 ± 0.037	0.005	1.4	0.064
H/D	20	0.528 ± 0.060	0.498 ± 0.056	−0.27	−6.0	<0.001*

C/W = 10°C, immersed; C/D = 10°C emersed; H/W = 25 C immersed; H/D = 25°C emersed.

TABLE 3.

Mean tissue uric acid concentration (μg g^{-1} fresh body weight) at timed intervals following emersion at 25 C.

Hours	N	Uric acid (mean ± SEM)	F	P
0	14	121.4 ± 14.9	—	—
3	15	167.0 ± 19.8	3.30	0.080*
6	15	197.0 ± 17.9	10.34	0.003*
18	14	223.1 ± 20.3	16.24	<0.001*
28	14	234.2 ± 21.4	18.62	<0.001*
48	14	279.5 ± 20.8	38.05	<0.001*

storage form, then one would expect it to be depleted once water becomes plentiful again. I have found this to be the case: re-immersion in seawater causes significant reduction in uric acid concentration. The fact that animals that were warm as well as dry had the highest concentration of uric acid, and then had the lowest concentration after immersion in warm water, further bears out the metabolic role of temperature. Once the metabolic pathways that degrade uric acid become active, they appear to proceed faster at a higher temperature than at a lower temperature. The temporary storage of uric acid avoids the loss of water that would accompany excretion of nitrogenous wastes in any form, but especially if the nitrogen is excreted in the form of ammonia.

Subjecting animals to desiccation has the possible effect of causing water loss, which would cause a relative rise in concentration of all materials in the body. Not only did the experiments reported here rule out any such effect on uric acid concentration (since the concentration of uric acid increased so much more than the body weight decreased), but the desiccation had a remarkably small effect on body weight. Even after 5 days in dry conditions, the animals only lost about 6% of their original weight, indicating the effectiveness of the opercular seal as well as the water sparing effect of uric acid storage.

Littorina saxatilis lives rather high in the intertidal zone. Therefore, these animals spend a great deal of time out of the water. Many individuals may be submerged only during spring tides. The time course for uric acid change during desiccation was shown to be sufficiently rapid to account for the changes in uric acid that probably occur during natural immersion/emersion cycles, especially during neap tides when many individuals may not be immersed for days, since the range in concentration I obtained within 48 hours approximates the range reported on a seasonal basis (Smith & Smith 1998).

It is interesting to consider what might serve as the signal to convert nitrogenous wastes to uric acid. Since the actual water loss was rather slight (about 6% after 5 days), and the increase in uric acid was significant within hours of emersion, it is unlikely that water loss can be the signal for the metabolic change. Winkles withdraw into their shell within minutes of water being withdrawn, and remain so until re-immersed. Possibly the same signal that stimulates withdrawal also serves to alter nitrogen metabolism. A possible stimulus for uric acid production could be a lowering of pH in emersed animals. When emersed individuals withdraw into the shell they become partially anaerobic in their metabolism. Anaerobic metabolism leads to a decrease in metabolism and perhaps stimulates uric acid production.

My results support the thesis of Eichelberg and Heil (1989) that winkles are not truly uricotelic if uricotelic is taken to mean being an excretor of uric acid. Winkles do synthesize uric acid as a water conserving means during times of emersion, but they apparently convert this uric acid back to ammonia in order to be excreted, and this only when water is again plentiful. Such enzymatic conversion of uric acid to ammonia should be possible, since Daguzan (1967) detected all the enzymes required for uricolysis in *L. littorea*, and it seems reasonable to expect that the same enzymes are present in *L. saxatilis* as well.

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THE EFFECTS OF EMERSION ON AMMONIA EFFLUX OF THREE HONG KONG *NODILITTORINA* SPECIES

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ABSTRACT On moderately-exposed Hong Kong shores, the nodilittorinids, *Nodilittorina trochoides* and *N. radiata* occur in the high-splash zone and *N. vidua* slightly below this level, extending into the eulittoral. These species experience long emersion times and high rock and air temperatures. After exposure on the natural rock for 0 min (awash and active, control animals) or 1, 4 or 22 h emersed, groups of animals (n = 6) were re-immersed and their ammonia effluxes (weight-normalized for fresh tissue mass) measured after 0.5, 1.0 and 2.0 h. Re-immersed groups had initial (30 min) effluxes of 3.91, 6.01 and 3.53 μmoles $NH_4 \times g^{-1} \times h^{-1}$ for *N. trochoides*, *N. radiata* and *N. vidua* respectively, which were high compared with the final values of 1.66, 2.02 and 0.32 μmoles $NH_4 \times g^{-1} \times h^{-1}$ for the same species after 2 h of re-immersion. There were clear inter-specific efflux differences and the handling procedure evoked enhanced ammonia excretion rates possibly as a stress response. Re-immersed animals had lower effluxes than control groups and, generally, such rates were negatively related to the duration of the preceding emersion period. Effluxes of *N. vidua*, measured 30 min after re-immersion were always higher than those measured at 1 or 2 h but this occurred with the other species only after they had been emersed for 1 h. Rates at all three sampling times following 4 or 22 h of emersion were very similar. The more eulittoral *N. vidua* may continue to produce ammonia during emersion periods of >1 h but the high-shore species do not. The energetic cost implications of this difference in post-emersion effluxes may be that emersion tolerance, hence vertical height on the shore, is limited for *N. vidua*. No evidence of a switch from ammonotely to uricotely was found for any species, but the production of non-excreted uric acid is not precluded.

KEY WORDS: Ammonia, effluxes, emersion, *Nodilittorina* spp., tropical rocky shore

INTRODUCTION

Three common *Nodilittorina* species occur high on Hong Kong rocky shores (Oghaki 1985, Reid 1992, Williams 1994, Mak 1996) and have littoral distributions that result in lengthy periods of emersion and, during neap tide cycles, individuals may be continuously emersed for some days (Williams 1994). Summer daytime temperatures in Hong Kong can be high and rock surface temperatures >50°C are not exceptional; conditions which enhance the risk of desiccation (see Williams and Morritt 1995, Williams and McMahon 1998). Each of the *Nodilittorina* species exhibit measures which are known to aid the maintenance of a balanced heat budget, including the development of a dried mucus "anchor" moving to forage while awash and ceasing activity shortly after becoming emersed (see Garrity 1984, Britton 1992). All three species are principally ammonotelic, a mode of producing and removing nitrogenous metabolic waste that is inexpensive energetically but which entails a loss of water that would be intolerable for a non-aquatic species. The fact that these nodilittorinids are numerous and dominate high shore regions in the tropics is clear evidence that they have evolved means to tolerate protracted periods when nitrogen metabolism continues but normal means of removing the ammonia produced are probably impractical because of the entailed water losses. As ammonia is toxic, it is of interest to know how these animals deal with their nitrogenous wastes at such times and a closer examination of the interspecific and intraspecific means used may improve our understanding of small-scale distributional differences shown by these, and similar, high shore invertebrates.

Several studies on the effects of emersion on bivalves have been made (e.g. Widdows et al. 1985, Schick et al. 1988, Sadok et al. 1999) which indicate considerable metabolic and behavioral variability can occur in these animals. Literature is limited on nitrogen metabolite fluxes of intertidal gastropods in general and no information on such fluxes in *Nodilittorina* spp. could be found. Switching biochemical pathways so that an increased relative proportion of other forms of metabolic waste is produced is energetically an expensive option, were a species able to do this, but would effectively reduce water loss, hence desiccation stress, and thereby decrease the risks of associated toxicity or tonicity. Consequently, much of the emphasis of the study of nitrogenous waste removal in gastropods in general, and littoral species in particular, has concentrated on the relative rates of production, storage and release of uric acid in relation to ecological preferences and water conservation (Delauny 1931, Needham 1935, 1938). Terrestrial gastropods are principally uricotelic and it is logical to speculate that high-shore gastropods may have developed so that the proportions of ammonia and uric acid produced may be intermediate between the terrestrial and aquatic representatives. Smith and Smith (1998) found that summer levels of soluble uric acid concentrations in *Littorina saxatilis* were significantly higher than winter levels, partly because of higher desiccation levels, but they suggest, mainly because of increased protein catabolism rates in summer.

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Whichever nitrogenous metabolic end-products are produced, their rates of production are reflections of metabolic rate. Comparisons of nitrogen fluxes between emersed and immersed littoral animals are complicated because emersed animals eventually cease locomotory movements resulting in general levels of slow metabolism.

These preliminary studies on three *Nodilittorina* spp. were made to determine the weight-specific ammonia efflux rates of normal (immersed) animals and to examine whether post-emersion ammonia effluxes are influenced by the emersion duration. It is not known whether brief, but inevitable, handling procedures constitute a stress sufficient to alter normal effluxes or what the magnitude and duration of these changes may be. Consequently, the ammonia effluxes of groups of each of the three species were assessed in terms of time elapsed since being handled and of preceding emersion duration. As ammonia is the product of respiring tissue and because a large proportion of the total wet weight of each animal comprises the non-respiring shell, the relative weights of shell and flesh were determined using a representative sub-sample of each species. All effluxes given here are thus corrected to a tissue mass basis.

MATERIALS AND METHODS

Nodilittorina trochoides (Gray 1839), *N. radiata* (Eydoux & Souleyet 1852) and *N. vidua* (Gould 1859) were collected while awash on the falling tide at Cape d'Aguilar, Hong Kong (22°N, 114°E) in July 1996. Eighteen individuals of each species were individually transferred to Eppendorf tubes containing 1.4 ml of the ambient seawater as a control group (awash) and the time was recorded. Fifty-four individuals of each species were also collected and placed on rock above the high water mark immediately in front of The Swire Institute of Marine Science. After 1, 4 and 22 h of emersion, three sets of six individuals of each species, one animal per tube, were transferred to Eppendorf tubes containing 1.4 ml of ambient seawater and the time recorded.

For each set of animals, after 30, 60 and 120 min, six individuals of each species were removed from their tubes, damp dried, weighed to the nearest 0.1 mg and replaced on the shore at the appropriate tidal level. The labeled Eppendorf tubes with the water samples were frozen (−20°C) until required for analysis.

Separately, a sample (n = 54) of freshly caught animals of the three species were damp-dried, weighed (± 0.1 mg), killed in boiling water and the flesh removed from the shells which were re-weighed (± 0.1 mg). A fresh tissue weight vs. total wet weight relationship was determined for each species.

Water samples were analyzed for dissolved total ammonia ($NH_4 = NH_3 + NH_4^+$) and uric acid (as urate). Ammonia concentrations (as total volatile base, TVB) were quantified using a flow injection/gas diffusion technique (Hunter and Uglow 1993) using a 500-ml sample loop. Calculated ammonia concentrations were transformed to weight-specific effluxes (μmoles $NH_4 \times$ g flesh weight$^{-1} \times h^{-1}$). Uric acid concentrations were determined as urate using Sigma diagnostic kit No 685.

Data were analyzed using Two Way Analysis of Variance (ANOVA). When significant differences were detected, the Tukey multiple range test was applied to compare means. All statistical analyses were performed at the 5% significance level.

RESULTS

Tissue weight vs. total weight relationships for the three species show that in each case, the relationship was linear and thus allowed the estimation of flesh weight from a simple wet weight determination (Table 1).

The weight-specific ammonia efflux data of the freshly-caught, non-emersed (control) animals decreased progressively (Fig. 1), particularly during the first 30 minutes, such that the 120 min values were significantly less than those measured at 30 min ($P < 0.05$ in each case). Presumably, such decreases illustrate a progressive recovery from the brief, initial handling shock and that the 60 and 120-min values better represent the normal, emersed effluxes of the species. The animals were crawling when captured and the ammonia concentration in any extra-corporeal water that they may have had would have been similar to that of the ambient water. The apparently high initial effluxes shown by *N. radiata* are probably not a reflection of the general activity of this species when first caught as it was noticeable that *N. vidua* was much more active than the other two species. The 120-min effluxes also indicate that those of the most eulittoral *N. vidua* are significantly less ($P < 0.05$) than those of the two higher shore species. The data were uncorrected for losses that may have occurred due to bacterial consumption. Presumably, some losses will have occurred in this way in the 1 and 2 h samples, but such losses were negligible in seawater blanks and it is assumed, therefore, that such losses would not alter the main findings substantially.

The two highest zoned species, *N. trochoides* and *N. radiata* had ammonia excretion rates following 1 h of emersion which were either similar or slightly less ($P > 0.05$) than those of their control groups (Fig. 2a–b). Longer periods of emersion evoked re-immersion effluxes that were significantly lower than those of the control groups ($P < 0.05$ in all cases).

Quite a different pattern of post-emersion ammonia efflux rate was found to occur in the species with the lowest vertical distribution, *N. vidua* (Fig. 2c). The control value for this species was significantly ($P < 0.05$) less than those of the other two species and, after each of the three emersion periods, the reimmersed efflux rates were significantly higher than the control group in all instances except for the 120 min post 4 h emersion group. The 1 and 4 h emersed groups showed a pattern of progressively diminishing post-emersion efflux rates but the 22 h emersed group maintained a reasonably constant, high efflux (*c.f.* the control group). None of the animals excreted measurable amounts of urate during these experiments.

The 120-min efflux data were analyzed using Two way ANOVA which revealed no significant differences due to species or emersion duration, but a significant species × emersion time interaction, suggesting that the rates of re-immersion adjustment differed between species. Tukey tests revealed that the mean efflux of freshly-captured *N. radiata* was significantly different from that of freshly-captured *N. vidua* and that the mean effluxes of the 22 h emersed *N. vidua* were significantly higher than those of 22 h emersed *N. trochoides* or *N. radiata*.

TABLE 1.

Relationships between total wet weight (X) and fresh tissue weight (Y) for 3 Hong Kong *Nodilittorina* spp.

Species	Regression details
Nodilittorina vidua	Y = 0.127X + 0.004 r = 0.9298
Nodilittorina trochoides	Y = 0.159X + 0.001 r = 0.8500
Nodilittorina radiata	Y = 0.093X + 0.002 r = 0.7300

n = 54 in all cases.

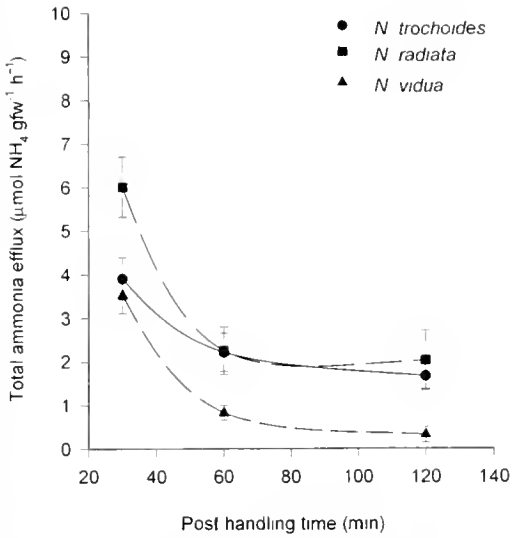

Figure 1. Weight-specific ammonia efflux of 3 species of *Nodilittorina* at various times following a brief handling procedure. Values are presented as means ± S.E. for separate groups of (n = 6) animals in each case. (T = 29.4 C, S = 30).

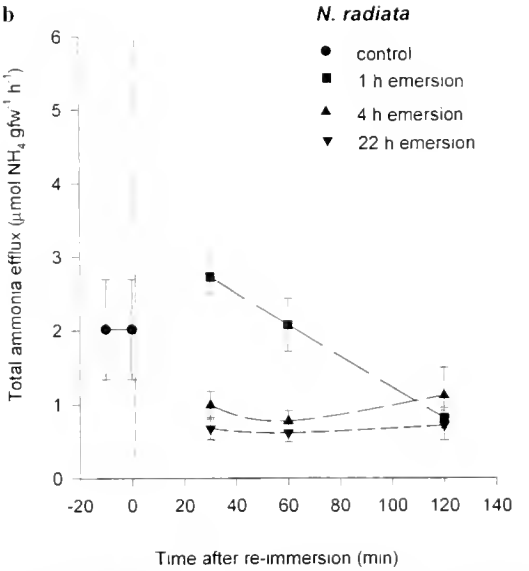

Figure 2b. Weight-specific ammonia efflux of *Nodilittorina radiata* at various post reimmersion times following emersion of 1h, 4h or 22h. Values are presented as means ± S.E. for separate groups of (n = 6) animals in each case. (T = 29.4 C, S = 30).

DISCUSSION

These preliminary findings reveal that considerable interspecific and intraspecific variability of ammonia efflux occurs amongst these littoral gastropod species and that some of the variability can be attributed to the positions that the animals are found on the shore. Brief handling and emersion evoked high ammonia effluxes after being handled and emersed for a very short period of time. These "shock" response rates persisted for at least 30 minutes and possibly, 120-min rates better represent the "normal" immersed, active effluxes of these species (Table 2). The control rates used here were those measured at 120 min following the brief handling procedures and were 1.66 ± 0.29, 2.02 ± 0.68 and 0.32 ± 0.08 μmoles $NH_4 \times g^{-1}$ flesh weight $\times h^{-1}$ respectively for *N. trochoides*, *N. radiata* and *N. vidua*. Ammonia has an oxycalorific value of 0.0689 cal × μmole^{-1} (Brafield and Solomon 1972) and, on the broad assumption that *N. trochoides*, *N. radiata* and *N. vidua* were immersed for 4, 5 and 12 h × d^{-1} respectively at the collection site, the daily calorific loss through this route when immersed would amount to 0.46, 0.70 and 0.26 cal × g^{-1} × d^{-1} for the three species. The equivalent ammonia losses would be 6.64, 10.10 and 3.84 μmoles $NH_4 \times$ g flesh weight^{-1} × d^{-1}. Duerr (1968) found no urea produced but 0.3–6.0 μmoles $NH_4 \times$ g wet tissue^{-1} × d^{-1} produced by seven species of marine prosobranchs and Crisp

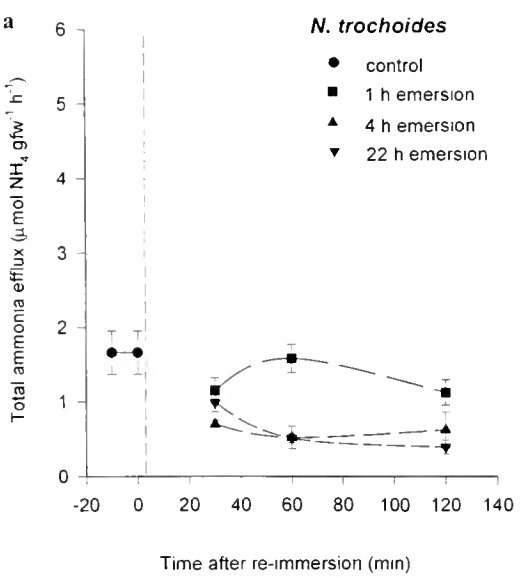

Figure 2a. Weight-specific ammonia efflux of *Nodilittorina trochoides* at various post reimmersion times following emersion of 1h, 4h or 22h. Values are presented as means ± S.E. for separate groups of (n = 6) animals in each case. (T = 29.4 C, S = 30).

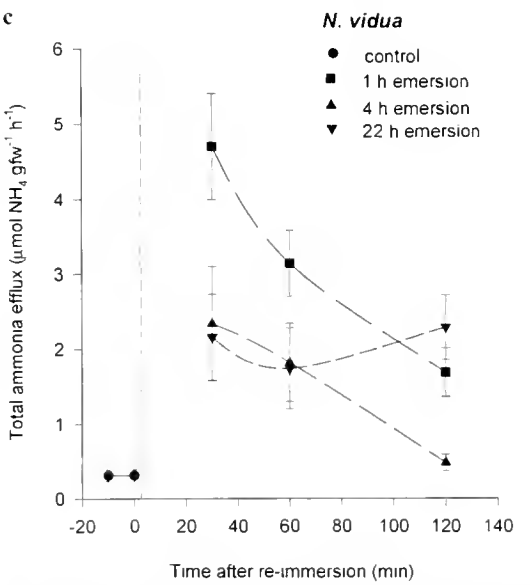

Figure 2c. Weight-specific ammonia efflux rates of *Nodilittorina vidua* at various post reimmersion times following emersion of 1h, 4h or 22h. Values are presented as means ± S.E. for separate groups of (n = 6) animals in each case. (T = 29.4°C, S = 30).

TABLE 2.

Ammonia efflux (μmoles $NH_4 \cdot g^{-1} \cdot h^{-1}$) of 3 *Nodilittorina* species collected *in situ* at high tide whilst awash and given no emersion.

Measurement period (min)	*N. trochoides* Mean ± S.E.	*N. radiata* Mean ± S.E.	*N. vidua* Mean ± S.E.
30	3.91 ± 0.48	6.01 ± 0.69	3.52 ± 0.42
60	2.21 ± 0.44	2.25 ± 0.55	0.83 ± 0.17
120	1.66 ± 0.29	2.02 ± 0.68	0.32 ± 0.08

Groups of n = 6 animals were used in each case (T = 29.4°C, salinity = 30).

et al. (1981) found that control group animals of the carnivorous gastropod, *Nassarius reticulatus* excreted ca. 0.76 μmoles $NH_4 \times$ g wet tissue$^{-1} \times h^{-1}$ (= 18.20. d^{-1}). Further studies will refine these estimates and will include estimates of the volatilised ammonia excreted during the emersion periods and the variability corrected for actual duration of the emersion periods experienced during the various tidal cycles at the collection site.

Clearly, the ammonia excretion rates following emersion vary according to species and the duration of the emersion period. After 1 h of emersion, the subsequent efflux rates for the first 30 min were as high (*N. radiata*) or higher (*N. vidua*) than the relevant control values. This suggests that ammonia production in these species had not only continued over this emersion period, though possibly at a lessened rate, but that it had been stored in a form and a site such that it was rapidly available for excretion as ammonia. During the 30 min following re-immersion after all the emersion times tested, *N. vidua*, excreted ammonia at rates which exceeded their control values but, in *N. radiata* showed this behavior after the 1 h emersion period only and *N. trochoides* did not show this behavior at all. These interspecific differences may relate to normal tidal distributions as the more eulittoral *N. vidua* will spend relatively much less time emersed than the high-shore *N. trochoides*. Consequently, the trade- off between the need to conserve water and to tolerate supra-normal ammonia concentrations is less critical in eulittoral species than for high shore species. Some of the post-emersion ammonia efflux that occurred in *N. vidua* and *N. radiata* may have been attributed to the flushing of ammonia-enriched mantle cavity water—but this was unlikely to have accounted for the entire amount. Similar occurrences have occurred with virtually all the crustacean and mollusc species we have examined and the source of this rapidly expelled ammonia is still under investigation. They are distinct from the effluxes derived from the general metabolic increase derived from handling.

The facility to reduce or cease ammonia production during periods of anaerobiosis is probably a prerequisite to successful colonisation of the high littoral levels where, at and above mean high water level at Spring tide (MHWST), prolonged emersion events are frequent. Additional studies will be needed to examine more critically the interspecific variability of emersion-dependance of volatilised ammonia losses and of uric acid production. No uric acid was found to be excreted in the 22 h emersions used in these tests but the production and storage of uric acid during emersion periods of this length are not precluded and such storage has been shown to vary seasonally in *Littorina saxatilis* (Smith and Smith 1998). The freshly-caught specimens of all three species showed an enhanced ammonia efflux following brief handling but this response is abolished if the handling is preceded by emersion for 4 h or more in *N. radiata* or for 1 h or more in *N. trochoides* but persists in *N. vidua*. In the last example, the rapidly excreted ammonia could represent the release of that produced during emersion and stored at some site such as the mantle cavity reservoir. In this instance, such reservoirs act less as a means of ensuring a modicum of aerobiosis during emersion, than as a latrine to which potentially toxic wastes can be consigned until they are flushed away on re-immersion. The techniques used appear to be suitably sensitive to allow accurate estimates of nitrogenous effluxes to be measured using the small volumes of water, or other fluids, which are often all that are available for analyses. They thus lend themselves to better estimations of energy fluxes in these types of animals, whether emersed or immersed, and to complement oxygen consumption studies which, often, are technically difficult, expensive or not feasible to use for certain types of energy flow studies.

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TRANS-ZONAL MOVEMENTS IN WINKLES, *LITTORINA LITTOREA* (L.): REASONS AND CONSEQUENCES

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ABSTRACT Two years of monthly samples of *L. littorea* at three levels on an estuarine shore at Southampton, U.K. have provided evidence of long-term movements up and down the shore. Increased population densities at the middle level of winkles in their second year of growth indicate movements of young winkles toward the center of their zonation range. Increases in population densities of older winkles at the higher and lower levels indicate later dispersion away from the center of the zonation range. Evidence of movements between the middle level and the lower level is provided by the presence or absence in the shell of bore-holes made by the polychaete *Polydora ciliata* (Johnston) which recruits mainly on the lower shore. At the upper level, fluctuating population densities and observations of feeding fronts in the spring indicate seasonal migrations, down in winter and up in spring. Advantages and disadvantages of living at upper or lower levels are discussed and related to differences in growth rates and predation risk.

KEY WORDS: *Littorina littorea*, movement, migration, zonation, age

INTRODUCTION

Littorina littorea is one of the best known members of the Littorinidae (Fretter & Graham 1994, Reid 1996, McQuaid 1996a, b). It is a widespread and common species both regionally and locally, occurring on both sides of the North Atlantic on a variety of substrata. Its preferred habitat is toward the middle and lower zones of sheltered rocky shores but the precise zonation varies somewhat from place to place (Underwood 1973) and extends from the upper regions of some shores into the sub-littoral of others. *L. littorea* also occurs on muddy and estuarine shores where there are pebbles and shells to cling to (Moore 1937, Warner 1997).

There have been many studies on populations of *L. littorea* that have produced a variety of conclusions concerning settlement and subsequent movements. Some authors observed settlement within the zone occupied by the adults (Moore 1937, Williams 1964) while others have reported sub-littoral settlement followed by upshore migration of juveniles during their first year of life (Smith & Newell 1955, Lambert & Farley 1968). Seasonal migrations up the shore in the spring and down in the winter have also been observed (Lambert & Farley 1968, Williams & Ellis 1975). In apparent contrast, short-term maintenance of zonation by "homing" of individual winkles has been reported (Newell 1958, Gendron 1977) as well as short-term "random" movements (Petraitis 1982). In some cases, differences in zonation of the various size-classes have been observed in which larger winkles occurred more commonly on the lower shore (Moore 1940, Smith & Newell 1955, Williams 1964, Lambert & Farley 1968, Fish 1972, Gendron 1977, Warner 1997). Little explanation has been offered other than that the lower shore may be "optimal" for *L. littorea* (Williams 1964). However if this were so, one would expect more winkles of all sizes to live there.

In the work reported here, individual ages of *L. littorea* were estimated by counting annual growth rings on the shells (Black 1973). Growth check marks may be caused by a variety of factors (Ekaratne & Crisp 1982), but at this site annual growth rings are prominent and appear to result from the temporary slowing of growth from late winter to May (Warner, in prep). Using these age estimates, age-specific zonation patterns were investigated. These patterns, and additional observations, are used here to infer orientated movements and as a basis for discussion of the balance of costs and benefits of living in any particular zone.

METHODS

The study site was at Netley on the northern shore of Southampton Water, which is an elongated, sheltered inlet on the south coast of England, receiving input from several rivers. The surrounding land is urbanized and industrial with considerable shipping activity. The tidal range is up to 5 m and the salinity is normally about 30‰. Wide mud and gravel flats are exposed at low spring tide, the distance from high to low water being about 300 m. Three sampling sites were established (Warner 1997). The upper site was close to mean tide level at 2.7 m above Chart Datum, the habitat was damp stony gravel with sparse *Enteromorpha* sp. and *Fucus* sp.; this site was chosen as the highest site where a sample of winkles could reliably be collected at all times of year. The middle site was 80 m further down the shore at 1.4 m above CD where the substratum was wet muddy sand with gravel on which grew a mixed algal community including *Chondrus crispus* Stackhouse. The lower site, close to MLWS, was a further 80 m down the shore at 0.7 m above CD where the substratum was wet mud with stones and shells and a sparse algal community similar to that at the middle site.

Samples of *L. littorea* were collected at approximately monthly intervals at each site from November 1995 to March 1998. Samples were collected within 50 × 50 cm quadrats, all winkle shells being collected from 2 or more quadrats at each site until more than 30 live individuals had been collected. 3,218 live winkles were collected in total (there was no indication that this regular removal of snails had any effect on the population on this extensive beach). All dead winkle shells were also collected from within the quadrats from January 1996. Winkles were killed by boiling (ca 30 sec) soon after collection and were extracted from their shells. The shells were dried and scored for the occurrence of epifauna, especially for the presence of *Polydora ciliata* (Warner 1997). Epifauna were scraped off and the length of the shells

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measured with calipers to an accuracy of 0.1 mm. Annual growth rings (Black 1973) were recognized as prominent growth check marks on the body whorl separated by several mm of shell growth. New annual growth rings were added in May/June of each year as new rapid growth recommenced following spawning (Warner, in prep). Winkle shells at this site become progressively eroded (Warner 1997), making younger and older sections of shell easily distinguishable, and accentuating the ring in between. Annual growth rings were counted to estimate age at 2+ years and above; O+ and 1+ shells showed no annual growth check but these cohorts were distinguishable by non-overlapping size ranges. It was assumed that each new year of life started at the end of May (the end of the spawning season — Williams 1964, Grahame 1975; personal observation at this site).

Additional information on winkle dispersion and movements in the upper part of the zonation range was collected just above the upper site in the vicinity of a line of aggregated winkles, parallel to the shoreline, which was observed in May and June of each year. This line coincided with the lower edges of wide patches of the green alga *Enteromorpha* growing on the pebbles. In 1997, winkle numbers were counted on a 4 m long transect of 16 contiguous 25 × 25 cm quadrats positioned normal to this line. In 1998, the position of the line of winkles was marked with 4 pegs 10 m apart and revisited twice at 14-day intervals; on each visit the position of the line in relation to each peg was measured. Aspects of mortality were studied by collecting dead shells as described previously. These were measured and examined for damage resembling that caused by crab predation (large, irregular holes in the body whorl or spire — verified by personal observation of green shore crab, *Carcinus maenas* (L.), predation on winkles in aquaria) and for bore holes of *P. ciliata*.

RESULTS

Relative Movements of Different Age Classes

The distributions of the various ages at the three levels on the shore, summed over the entire sampling period and expressed as percentages, are shown in Figure 1. These age distributions are very significantly different from each other (contingency test, $\chi^2 = 295$, d.f. = 14, $p = 0.000$), showing an association between level and age. The differences include proportionally fewer age 1+ winkles at the upper and lower sites, and more 1+ winkles at the middle site. There were also proportionally fewer 3+, 4+ and 5+ winkles at the middle site, more 2+ and 3+ at the upper and more 3+, 4+, and 5+ winkles at the lower site. These data could be explained by movement of 1+ winkles toward the middle site, mainly from lower locations, and dispersal of older winkles from the middle site both up and down the shore. Total winkles collected at any one age increased from 0+ to 2+, then declined, but winkles estimated at more than seven years old were found at all sites. Winkle density was much higher at the middle site than at the upper and lower sites (Table 1).

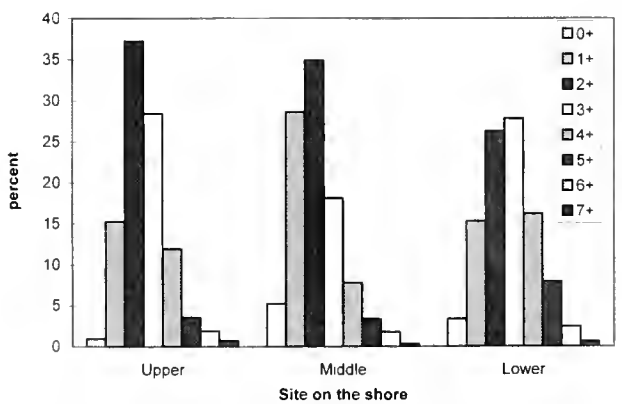

Figure 1. The percent frequency of different age classes of *L. littorea* at three sites on the shore, showing an association between level and age (contingency test on raw data, $\chi^2 = 295$, d.f. = 14, p = 0.000). At the middle site, there were relatively higher proportions of younger winkles (age 1+ years), and lower proportions of older winkles (ages 3+ and 4+ years).

Indirect evidence for movement of juvenile winkles toward the middle of their zonation range, and of older winkles away from the middle, was also provided by a biological marker: the bore holes of *P. ciliata*. Examination of live winkles showed *P. ciliata* holes in a proportion of shells at all levels (Table 1). However, infection was high at the lower site and much lower elsewhere, leading to the conclusion (Warner 1997) that settlement of *P. ciliata* is mainly restricted to the lower shore. The pattern of *P. ciliata* infection of winkles is that the worms prefer to settle on older areas of shell which lack periostracum (Warner 1997). In 0+ winkles, no *P. ciliata* borings were found at any site. In 1+ winkles, the only area of shell which is "old" and thus suitable for settlement is the relatively small area of the spire, since the whole body whorl is young and smooth. This leads to a characteristic pattern of infection in juveniles on the lower shore in which borings are often found only in the spire. Figure 2 shows the percent distribution of *P. ciliata* infection in winkles of different ages from the lower and middle sites (the upper site had too few bored winkles for feasible analysis). In both cases, there is a highly significant association between age and bore-hole distribution (contingency tests, lower: $\chi^2 = 203$, d.f. = 6, $p = 0.000$, middle: $\chi^2 = 79$, d.f. = 6, $p = 0.000$, data for >4+ snails contained a zero so was excluded). At the lower site, it is clear that borings only in the spire were common in 1+ winkles. Figure 2 shows that this pattern is gradually eliminated later in life by borings distributed elsewhere on the shell. At the middle site, 1+ winkles with *P. ciliata* borings only on the spire were not common, but a small proportion of 2+ winkles showed this pattern. These may have been individuals that were lower down the shore at an earlier age and moved up at about 1+ years old. In contrast, the relative increase in proportions of unbored 2+ and 3+ winkles at the lower site, shown in Figure 2, provide evidence of downshore movements in older snails.

TABLE 1.

Mean densities at the three sampling sites of live winkles and dead winkle shells, and the percentages of each of these that were bored by *P. ciliata*. Also included are the percentages containing bore-holes of those dead shells that appeared to have been cracked by crabs.

	Upper site	Middle site	Lower site
Live per m^2 ± SD	54.1 ± 28.7	116.7 ± 25.1	41.1 ± 10.8
Dead per m^2 ± SD	8.3 ± 5.7	17.9 ± 13.7	4.8 ± 2.6
% live + bore-holes	4.7	10.2	63.5
% dead + bore-holes	8	35.5	84
% cracked + bore-holes	35	76	97

Calculated from all samples (n = 30 at each level) collected between 1/96 and 3/98.

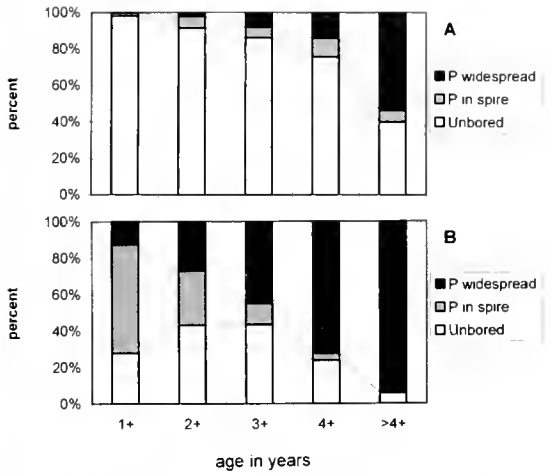

Figure 2. Distribution of bore-holes of the polychaete *Polydora ciliata* on the shells of different age-classes of live *L. littorea* at two levels on the shore, showing that the incidence of boring increases with age, and that on the lower shore, boring in the spire precedes more widespread boring (contingency tests on raw data, lower: $\chi^2 = 203$, d.f. = 6, p = 0.000, middle: $\chi^2 = 79$, df = 6, p = 0.000; data for >4+ snails contained a zero so were excluded). A = middle site, B = lower site; P = *Polydora*.

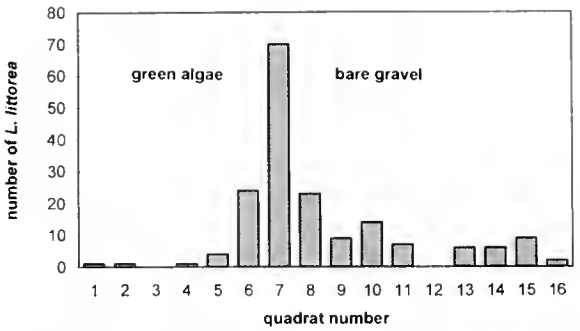

Figure 3. Results of a 4-m long transect, normal to the shoreline, of contiguous 25 × 25 cm quadrats on the upper part of the shore in May 1997, from gravel covered with green algae (higher) to bare gravel (lower, total drop ca. 5 cm), showing a high density of *L. littorea* forming a feeding front at the boundary of the algal growth.

Seasonal Migrations

In May and June, just above the upper site, a line of aggregated winkles parallel to the shoreline was observed to coincide with the lower edges of wide patches of the green alga *Enteromorpha* growing on the pebbles. Figure 3 shows the results of the 1997 transect across this line. In 1998, the line was found to move upshore by a mean distance of 103 cm in 14 days (n = 8, range = 45–169 cm), the winkles having apparently grazed the *Enteromorpha* leaving bare clean pebbles behind. By July, these "feeding fronts" had dispersed but winkles were still present above the upper site, often clustering at low tide beneath sparse clumps of *Fucus*. By November, winkles became rare above the upper site. These observations indicate upshore migration occurring in spring and summer. Since there were no clear seasonal changes in numbers of dead shells, downshore movements presumably occurred in autumn. Fluctuations in monthly population density measurements at the upper site were much greater than at the other two sites (Table 1) and appeared to correlate roughly with the seasons. The lowest densities were recorded between October and March (mean 35.3 m^{-2}, SD = 21.1, range = 7–65, n = 14) and the highest between April and September (mean = 65.6 m^{-2}, SD = 29.7, range = 31–148, n = 16). These means are significantly different (two-sample *t*-test, $t = -3.25$; d.f. = 27; $P < 0.01$).

Some Costs and Benefits of Life on the Lower Shore

Data on growth rates collected during this study show that growth was faster at the lower site than at the other two sites. Winkles of the same age were larger at the lower site, and showed greater annual growth increments at the shell margin than those from higher up the shore. These data will be published elsewhere.

The mean percent of dead shells relative to total (live + dead) shells from the different sites are shown in Figure 4A and are not significantly different. These data show that dead shells occur in proportion to the live population, and suggest that mortality rate is similar throughout the shore, despite differences in age distribution (it was assumed that dead shells "survived" to be sampled equally at the three sites). Figure 4B shows the mean percent of dead shells at the three levels that appeared to have been cracked by crabs. These proportions are significantly different (Single factor ANOVA, $F = 11.9$, d.f. = 68, $p = 0.000$): cracked shells were far more common at the lower site than at higher sites. Table 1 shows the incidence of *P. ciliata* bore-holes in the samples of dead and live shells from the 3 sites. It may be seen that while the incidence of bore-holes in both live and dead winkles decreased up the shore, a consistently higher proportion of dead shells at all sites contained bore-holes, and this proportion was higher still in those dead shells presumed cracked by crabs. The chi-square contingency tests on the raw data showed significant, positive association between being dead and the incidence of bore-holes at both the middle and lower sites ($\chi^2 = 100.7$ and 17.6 respectively, d.f. = 1, $p = 0.000$). Similarly, among dead shells at all three sites there was significant, positive association between the incidence of bore-holes and being cracked ($\chi^2 = 6.6$–23.8, d.f. = 1, $p < 0.01$).

DISCUSSION

Some previous work on *L. littorea* has shown that larvae settle on the shore throughout the zone of the adults (e.g. Williams 1964,

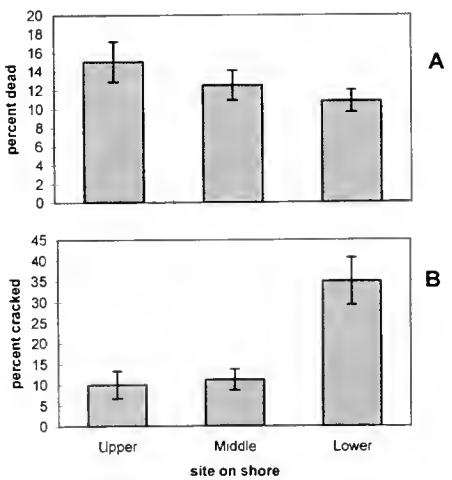

Figure 4. The mean frequencies (±SE) of dead (as percent of live + dead) *L. littorea* at three sites on the shore: not significantly different. B. The mean frequencies (±SE) of cracked (as percent of total dead) *L. littorea* at three sites on the shore, showing a greater proportion cracked at the lower site (single factor ANOVA, F = 11.9, d.f. = 68, p = 0.000).

Underwood 1973), but other authors (e.g. Smith & Newell 1955; Gardner & Thomas 1987) concluded that settlement is mainly sublittoral and is followed by upshore migration. Part of the disagreement appears to be due to the difficulty of finding very small winkles. This problem was evident in the present work, with relatively few 0+ winkles being found at any site (Fig. 1). However, small (5–7 mm) 0+ winkles appeared at about the same time in autumn in the samples at all three sites suggesting that they had been there, unobserved, since settlement. The mixed nature of the substratum at Netley provided many hiding places for tiny winkles (between pebbles, amongst algae, etc). Settlement at Southampton is therefore probably littoral rather than sublittoral, but the data for 0+ winkles (Fig. 1) suggest that recruitment was more frequent in the middle of the zonation range (middle site) than at the upper site at mean tide level.

In older winkles, changes in population density at a site must be due to movements of snails between zones or to differential mortality of snails. Smith & Newell (1955) and Williams (1964) found evidence of an upshore movement of young winkles (first and second year respectively), and Smith and Newell (1955) speculated that a downshore movement of older winkles could account for the presence of larger ones on the lower shore. The demonstration here of an excess of 1+ winkles at the middle site (Fig. 1) suggests active movement of young winkles toward the middle of the zonation range from both lower and higher levels. Smith and Newell (1955) ruled out the alternative of differential mortality of young snails on the grounds that dead shells were found distributed in proportion to the living, and this was also found here (assuming that dead shells were not unequally moved, fragmented or buried at the different levels). Similarly, subsequent dispersal seems the only plausible explanation of the increasing proportions and densities of older age-classes at the upper and lower sites. The data from *P. ciliata* borings (Fig. 2) provided evidence of both the movement upwards of juveniles from the lower shore and the movement down of age 2+ and 3+ winkles from the middle of the zonation range. However, it should be clear from these data that the full extent of trans-zonal movements were not performed by all individuals; winkles of all age groups remained present at all sites, indicating that some moved less than others.

Age-class specific movements may be thought to conflict with movements that have been described as maintaining the particular zones of individuals (Fretter & Graham 1994). These include small-scale circular movements (Newell 1958) and "home" orientated movements following displacement (Gendron 1977). However, the movements described here did not involve all winkles and occurred over a time scale of months to years, thus they can coexist with short-term maintenance of individual shore level, and with the short-term "random" movements described by Petraitis (1982). They also coexist with the winter downshore and spring upshore "migrations"of winkles (Lambert & Farley 1968, Williams & Ellis 1975) which have been observed only in the upper part of the zonation range.

The question remains: What functions do these movements perform in the life of the winkle? Movements in early life toward the middle of the zonation range may serve a "habitat location" function. Movement of older adults away from the center may then be a response to high population density, serving to reduce competition. However, movement up has very different consequences from movement down. Movement up gives access to the spring growth of green algae but also entails an annual retreat from upper levels as temperatures fall in the winter. Movement down brings the benefit of faster growth, probably due to longer immersion allowing longer feeding periods. Since faster growth leads to larger size, it may also lead to greater fecundity in lower shore individuals. However, life on the lower shore also entails increased risk of predation from crabs (Fig. 4) and increases the variety of epibiota, including *P. ciliata*, which settle on the shell (Warner 1997). Almost all dead, cracked (i.e. crab-predated) shells on the lower shore were bored by *P. ciliata* (Table 1) suggesting that borings may increase predation risk by weakening the shell (Buckley & Ebersole 1994). *L. littorea* has few defences against epibiota, some of which have adverse effects through increasing drag on the snails (Wahl & Sönnichsen 1992, Wahl 1996). Thus, there are several reasons for not regarding the lower shore as "optimal" for this species (Williams 1964), despite the faster growth that can be achieved there.

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CORRELATION OF MORPHOLOGICAL DIVERSITY WITH MOLECULAR MARKER DIVERSITY IN THE ROUGH PERIWINKLE *LITTORINA SAXATILIS* (OLIVI)

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ABSTRACT Two morphological varieties of *Littorina saxatilis*, widespread around the United Kingdom, are a thin-shelled, high-shore morph (*L. saxatilis* H) and a thick-shelled, mid-shore animal (*L. saxatilis* M). Mitochondrial DNA (mtDNA) analysis by PCR-RFLP was used to test whether gene flow between these morphs is restricted. At Galloway, Scotland, replicated sampling (different years and different transects over a distance of 800 m) has been undertaken. One mtDNA haplotype is predominant in H and a different haplotype common in the M animals. Repeatability in space and time suggests a real H/M differentiation. A similar pattern of mtDNA haplotype variation is seen in a single sample of the Swedish E and S morphs of *Littorina saxatilis*. However, this pattern is not evident everywhere. Variation in the mtDNA and four nuclear DNA loci was examined within and between *L. saxatilis* H and M morphs from the south coast of England. Because shape variation in this region additionally separates into three shape groupings (regions identified from multivariate morphometric analysis where shape is more homogeneous within, than between groups), genetic variability was examined within and between these groupings as well as between H and M. On the south coast, an apparent association of shape and mtDNA haplotype is identified, but AMOVA analysis shows no support for the association being with shape grouping or H and M morphs. Although the nature of this shape-genotype association is unknown, a mtDNA haplotype and an allele at the nuclear CAL-2 locus are confined mainly to one shape group. Analysis of association of mtDNA haplotype with H and M morphology suggests a strong correlation can be found in some areas (Galloway, Mumbles (south Wales), and between similar morphs in Sweden) yet no association is seen at others (Ravenscar, UK, Ballynahown, Ireland, and the south coast of England). Thus, unravelling the basis of the H and M forms will require more detailed studies, with replication, as at Galloway, and also with additional molecular markers.

KEY WORDS: AMOVA, *Littorina saxatilis*, mitochondrial DNA, morphometrics, PCR, RFLP, shape

INTRODUCTION

Shell shape in gastropod mollusks is known to be affected by environmental factors. Selection as a result of crab predation or stone damage can produce heavy shells with small apertures, while animals in exposed conditions where crab damage is minimal, but risk of dislodgement by waves is high, tend to have thin shells with wide apertures (Boulding 1990). In *Littorina saxatilis* (Olivi) two forms typical of these morphological extremes are widespread. Hull et al. (1996) referred to the thin-shelled, patulous form, found in high-shore areas as *L. saxatilis* H, and the thick-shelled variant that occurs in the mid-shore as *L. saxatilis* M. Although the basis of this shell polymorphism undoubtedly has a strong environmental component, evidence from embryological characters (Hull et al. 1996) and detection of assortative mating for these morphotypes (Hull 1998, Pickles & Grahame 1999) suggests that there may be a genetic component to the differentiation of *L. saxatilis* H and M. However, the only molecular level study of genetic differentiation between these forms was limited to two sites on the east coast of Yorkshire, England. Random amplified polymorphic DNA (RAPD) analysis showed differing degrees of differentiation at these two sites (Wilding et al. 1998). No wide scale geographic studies have yet been undertaken. This is important since such evidence is necessary to determine the extent of gene flow between these morphs, which is critical for the understanding of their relationships.

Mitochondrial DNA (mtDNA) is a useful molecular marker for such a study because of its high rate of evolution (Avise 1994), and a substantial portion of the *L. saxatilis* mitochondrial genome has been sequenced (Wilding et al. 1999), thus permitting the targeting of primers. Population genetic studies of gastropods have often relied on mitochondrial markers (Kirby et al. 1997, Hellberg 1998, Kyle & Boulding 1998) and Wilding et al. (2000a) have recently examined mtDNA cytochrome oxidase I (CoI) variation in UK and Irish *Littorina*. We also have developed primers for the amplification of four variable nuclear DNA (nDNA) loci in *Littorina saxatilis* (Wilding et al. 2000b).

Here shape variation and genetic variation are examined at four levels. (1) Between *L. saxatilis* H and M in replicated samples from southwest Scotland. (2) Between *L. saxatilis* H and M on the south coast (where nDNA variation is also examined). (3) Between *L. saxatilis* H and M at other locations. (4) Along the south coast of the United Kingdom where three 'shape groupings' are recognized. It is shown that both shape and mitochondrial DNA do vary geographically, but that there is only a limited correlation of the two. There is however evidence for differentiation at the mtDNA level between *L. saxatilis* H and M in some areas of the UK.

MATERIALS AND METHODS

Animals were collected from 17 sites around the UK (Table 1). Images of the shells were captured with a digital camera and used for morphometric measurements. Measuring of shell images was performed using Sigma Scan to measure eight shell variables (Fig. 1). These shell variables were standardized for size using the geometric mean transform (Reist 1985) and then submitted to a canonical variate analysis in SAS v 6.0 (SAS Inc. 1990). The shell was subsequently crushed and a portion of the head-foot removed for DNA extraction using the single-fly DNA extraction protocol of Ashburner (1989). DNA concentration was measured by fluorometry and adjusted to 10 ng.μl^{-1}. A 967 bp segment of mitochondrial DNA spanning the cytochrome oxidase I–cytochrome

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TABLE 1.

Collection sites for samples of *L. saxatilis* H and M. South Coast samples are grouped in the three shape zones used in some analyses.

Collection site	British National Grid Reference	*N* (H)	*N* (M)
South coast			
Kent			
St Margaret's at Cliffe	TR368444	10	10
Folkestone	TR245369(H) & TR244373(M)	10	9
South-central			
St Alban's Head	SY959754	10	
Portland Bill	SY675683	9	
East Fleet	SY799635	10	
The Fleet (gravel)	SY758665		10
Golden Cap	SY407918		20
Pinhay Bay	SY318907		5
South-west			
Cargreen	SX436627		10
The Lizard	SW699114	10	
Trevaunance	SW725519	10	3
Other locations			
Galloway			
St. Ninian's Cave	NW417364	48†	49†
Back Bay	NW368394	7	10
South Wales			
Mumbles	SS632873	10	10
North East England			
Ravenscar	NZ984021	10	10
Ireland			
Ballynahown	IGR: L 992202	10	10
Sweden			
Ursholmen	58°50′10″N 0°59″4′ E	9*	8*

N = sample size.
† replicated samples taken at Galloway, see Fig. 4.
* morphs in Sweden are E and S, not H and M—see text.
IGR = Irish Grid Reference.

oxidase II (CoI-CoII) gene boundary (Wilding et al. 1999) was then amplified using the primers saxCoI and saxCoII (Wilding et al. 2000a). PCR was performed in 50μl volumes containing 50 mM KCl, 10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin, 200 μM each dNTP, 25 pmol each primer, 25 ng DNA and 1U *Taq* (Super*taq*, HT Biotechnologies). PCR

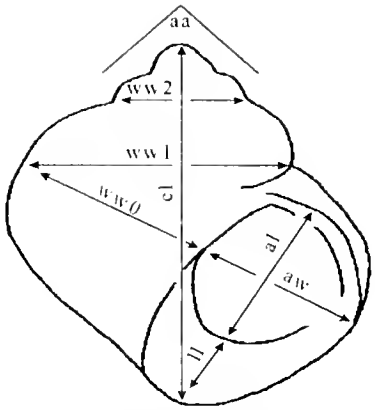

Figure 1. The eight shell variables measured in Sigma Scan. aa = apical angle. al = aperture length. aw = aperture width. cl = columellar length. ll = lip length. ww 0–2 = whorl width 0–2.

conditions of 1 × 94°C, 5 min; 35 × (94°C, 1 min, 55°C, 30 secs, 72°C, 1 min); 1 × 72°C, 5 min were employed. In an initial study, 400 bp of mtDNA were sequenced in 50 individuals from 18 populations (Wilding et al. 2000a). From the resultant sequences variable restriction sites were deduced. Variation was then assessed by RFLP using three restriction enzymes — *Dde*I, *Dra*I and *Hin*dIII (*Dra*I is not variable in *L. saxatilis* but is in other rough periwinkles). Restriction enzyme digestion was undertaken in 15 μl volumes containing 3-5U enzyme in 1X buffer (supplied with enzyme) and 5 μl PCR product. Following digestion, restriction fragments were separated on 2% agarose gels. Variant patterns generated by each enzyme were labeled A, B, C *etc.* and a three letter composite haplotype describes the variation in each animal. An analysis of molecular variance (AMOVA: Excoffier et al. 1992) was used to examine genetic variation from animals along the south coast of England. *Littorina saxatilis* in this region is known to exhibit substantial shape variation which has been shown to partition into three groups (Grahame and Mill 1992, Mill and Grahame 1995), a south-west, a south-central, and a Kent grouping (Fig. 2). Upon this broad-scale variation there is superimposed the H and M differentiation. AMOVA was used to quantify the partitioning of mtDNA variation into a 'shape grouping' component and a 'H and M' component. If substantial variability is accounted for by either of these then the expectation is that these shape differences are indicative of underlying genetic differentiation. If little genetic variability is partitioned into either of these compo-

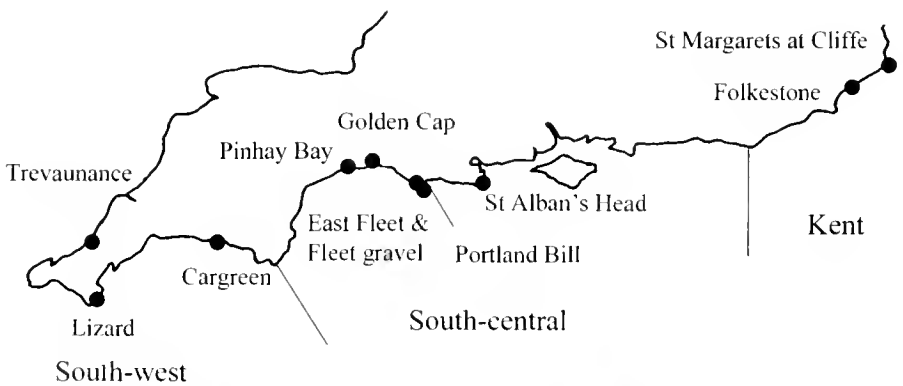

Figure 2. Delineation of shape groupings as described by Grahame and Mill (1992) and Mill and Grahame (1995).

nents then there is no detectable genetic differentiation at the mitochondrial DNA level between these groups. AMOVA was performed in ARLEQUIN v1.1 (Schneider et al. 1997).

In addition to the mtDNA analysis, patterns of nuclear DNA (nDNA) polymorphism have been examined in these animals (Wilding et al. 2000b). Four nuclear loci comprised of two calmodulin introns (CAL-1 and CAL-2), and two anonymous loci (X80 and DELETION) were examined for variation. All nDNA PCR reactions were undertaken in 25 μl of 50 mM KCl, 10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin, 200 μM each dNTP, 12.5 pmol each primer, 12.5 ng DNA and 0.5 U *Taq* (Super*taq*, HT Biotechnologies). PCR cycling conditions were 1 × 94°C, 5 min; 35 × (94°C, 1 min, 55°C, 30 secs, 72°C, 1 min); 1 × 72°C, 5 min, although for CAL-1 the annealing temperature was increased to 57°C, and DELETION in which annealing was at 57°C and the extension time was reduced to 30 seconds. CAL-1 and CAL-2 polymorphism was examined by *Taq*I and *Dde*I digestion respectively and in X-80 with *Msp*I. Restriction digestion was performed at the appropriate temperature for 2 hours and products were separated on 2% agarose gels. For the DELETION locus, alleles differed due to length variation and this was recognized after PCR products were run on 3% agarose gels. These nDNA data were not suitable for AMOVA analysis (because although the genetic distance between genotypes was calculable for those loci examined by RFLP, the mutational distance between alleles at the length variable DELETION locus was not. Thus the relationships among multi-locus genotypes, necessary for AMOVA analysis, were unclear). Correlation of shape variation with molecular variation was therefore examined using a Mantel test employed in NTSYS through comparison of the population matrix of genetic distance (different genetic distance measures were tested in case of subtle differences due to method employed) with the Mahalanobis distance matrix.

RESULTS

Mitochondrial DNA Variation

A neighbor-joining tree based on all sequences of CoI-CoII from *Littorina* shows two groupings of *L. saxatilis* (Fig. 3). Although there are 13 separate *L. saxatilis* sequences, these cannot all be distinguished using RFLP since there are many variable positions at this locus where there is no restriction enzyme with a recognition sequence spanning the site. However, three enzymes (*Hin*dIII, *Dra*I and *Dde*I) do recognize variable positions in this sequence within the rough periwinkles (Wilding et al. 2000a), although *Dra*I is not variable in *L. saxatilis*. In *L. saxatilis*, *Hin*dIII produced two patterns and *Dde*I four patterns. The four composite haplotypes encountered in *L. saxatilis* were designated AAA, ADA, BBA and BCA.

The most pronounced evidence for association of mitochon-

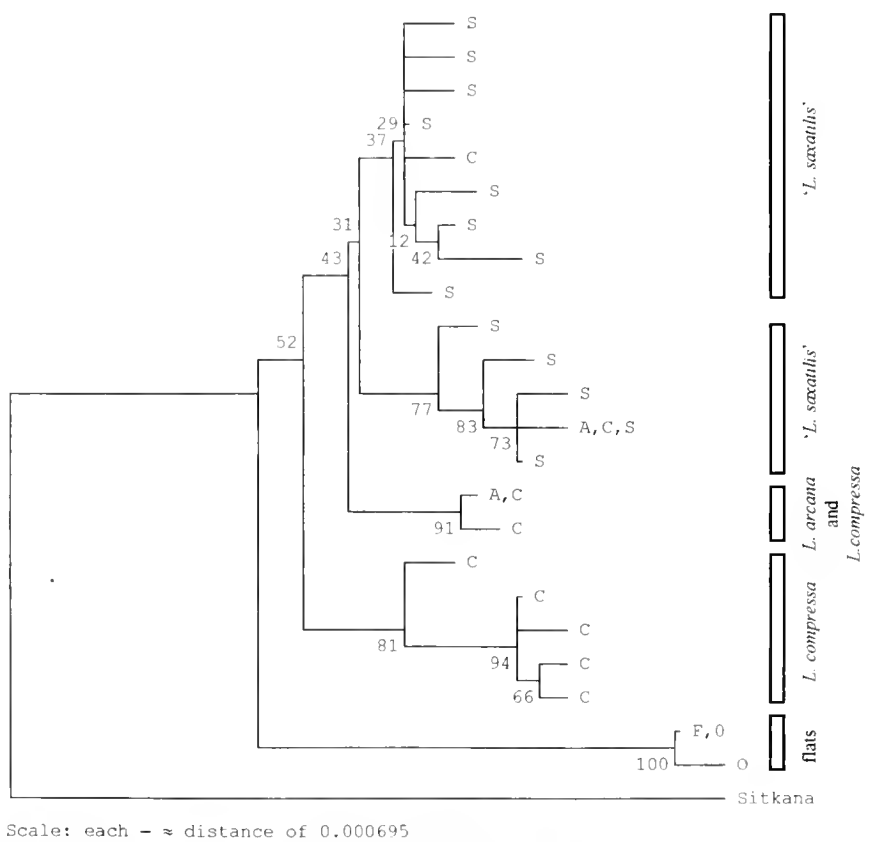

Figure 3. Phylogeny of rough periwinkle CoI sequences (adapted from Wilding et al. 2000a) using neighbor-joining of Jukes-Cantor distances. S = *L. saxatilis*, A = *L. arcana*, C = *L. compressa*, F = *L. fabalis* and O = *L. abtusata*. The two divisions of *L. saxatilis* sequences are denoted, note that some *L. orcana* and *L. compressa* sequences are shared with *L. saxatilis* or cluster in these "*L. saxatilis*" groups. Flats = sequences from the flat periwinkles *L. fabalis* and *L. obtusata*. The outgroup is *L. sitkana*. Numbers on the nodes are bootstrap values from 100 pseodoreplicates.

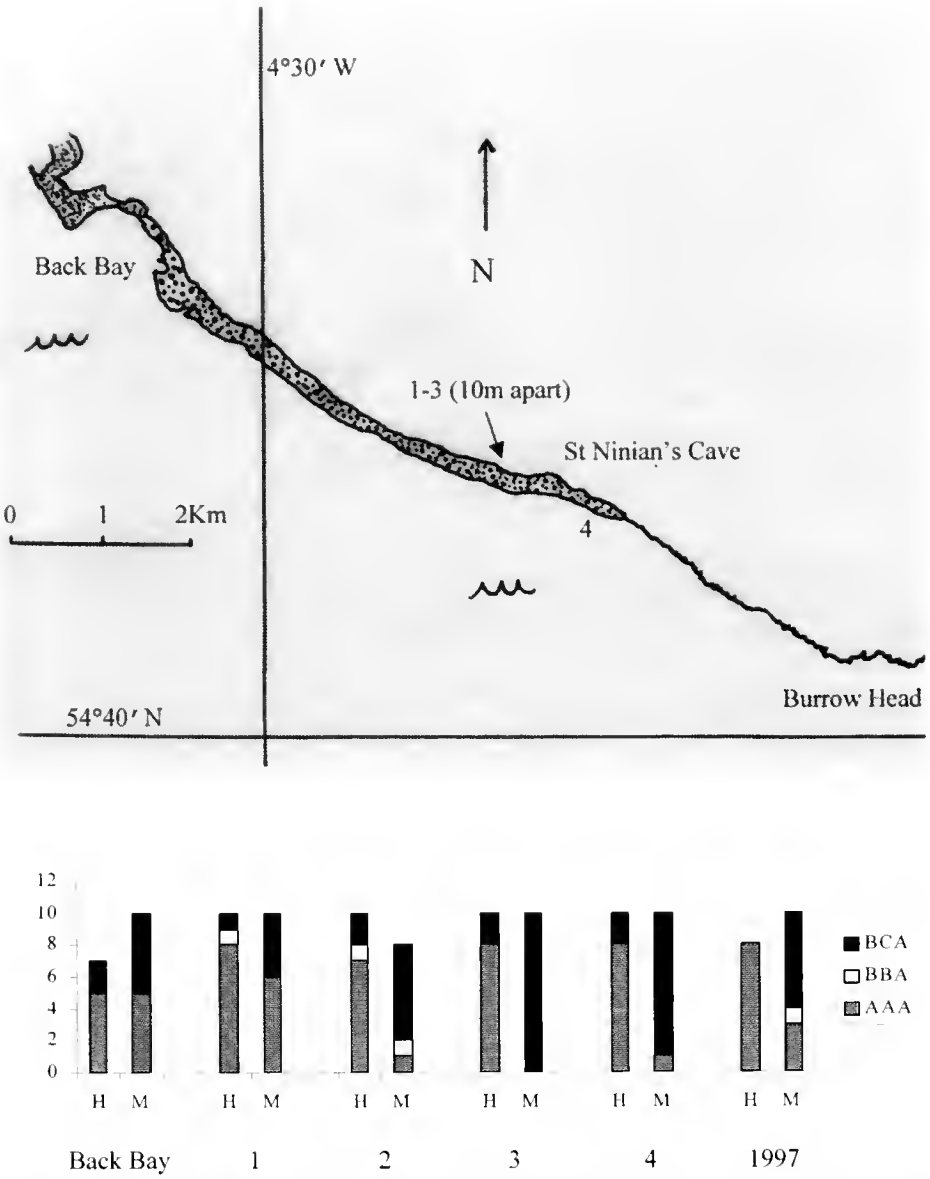

Figure 4. Distribution of mtDNA haplotypes in separate transects, undertaken in 1997 and 1998, at Galloway, southwest Scotland. Sites 1–3 were spaced 10 m apart and are separated from site 4 by approximately 800 m of shingle beach. The 1997 sample was taken from the same site as site 3 of 1998.

drial haplotype with morphology is found at Galloway in southwestern Scotland. Here one collection in 1997 and five collections in 1998 (from different transects) were typed. The AAA haplotype was predominant in *L. saxatilis* H and the BCA haplotype in *L. saxatilis* M (Fig. 4). Additional data from Mumbles (South Wales) also suggests differences between H and M, but at Ravenscar (east coast of England) and Ballynahown (western Ireland) no differences were detectable (Table 2). At Ursholmen, Sweden, differences were detected between the E and S morph of *L. saxatilis*. The E (exposed) morph, like *L. saxatilis* H, is wide apertured and thin-shelled and the S (sheltered) morph thick-shelled. However, unlike *L. saxatilis* H and M, where animals are separated vertically on the shore, E and S are separated locally, by habitat along the shore, being found in exposed and sheltered localities respectively. For the small sample examined, all nine E had the AAA haplotype, and 7 of 8 S had the BCA haplotype. These are the same haplotypes separating the H and M at Galloway.

South Coast of England — Morphological

Littorina saxatilis are shown to cluster into two groups on the basis of shell variation analyzed by canonical variate analysis (Fig. 5). When canonical variate means are plotted, the two groupings are associated with *L. saxatilis* H and M.

South Coast of England — Molecular

The distribution of haplotypes along the south coast within *L. saxatilis* H and M is shown in Table 2 and Fig. 6. There is sub-

TABLE 2.

Observed number of the 4 mtDNA haplotypes in the studied populations, and allele frequencies at the 4 nDNA loci for the south coast samples.

Collection site	MtDNA haplotype				CAL-1		CAL-2			X-80			Deletion		
	AAA	ADA	BBA	BCA	A	B	A	B	C	A	B	C	A	B	*N*
South coast															
South-east															
St Margaret's at Cliffe—H			10		0.45	0.55		1		0.2	0.8		0.3	0.7	10
St Margaret's at Cliffe—M	1		8	1	0.55	0.45		1		0.1	0.9		0.6	0.4	10
Folkestone—H			3	7	0.15	0.85		1		0.05	0.95		0.25	0.75	10
Folkestone—M	1		2	6	0.167	0.833		1			1		0.222	0.778	9
South-central															
St Alban's Head		1	9		0.6	0.4	0.4	0.6		0.15	0.85		0.35	0.65	10
Portland Bill		6	3		0.333	0.667	0.06	0.944			1		0.389	0.611	9
East Fleet		2	8		0.25	0.75		1		0.1	0.9		0.45	0.55	10
The Fleet (gravel)			10		0.4	0.6	0.1	0.9			1		0.3	0.7	10
Golden Cap		20			0.325	0.675	0.5	0.5		0.275	0.725		0.1	0.9	20
Pinhay Bay		5			0.8	0.2	0.8	0.2			1		0.4	0.6	5
South-west															
Cargreen	5			5	0.85	0.15		0.95	0.05	0.1	0.9		0.25	0.75	10
The Lizard		6		4	0.25	0.75	0.1	0.9		0.55	0.45			1	10
Trevaunace—H	1	1	6	2	0.4	0.6		1		0.2	0.75	0.05		1	10
Trevaunace—M			1	2	1			1		0.667	0.333		0.167	0.833	3
Other locations															
Galloway															
St. Ninian's Cave—H	39		2	7											48
St. Ninian's Cave—M	11		2	36											49
Back Bay—H	5			2											7
Back Bay—M	5			5											10
South Wales															
The Mumbles—H	3		5	2											10
The Mumbles—M			7	3											10
North East England															
Ravenscar—H	10														10
Ravenscar—M	10														10
Ireland															
Ballynahown—H	1		9												10
Ballynahown—M			10												10
Sweden															
Ursholmen—E	9														9
Ursholmen—S	1			7											8

N = sample size.

stantial variation within both H and M forms of *L. saxatilis* but no obvious difference in haplotypes between the two groups. However, there is some evidence for association of haplotype with shape grouping since the haplotype ADA is found mainly on the south coast in the south central group of shape variation. To investigate if haplotype partitioned with shape, haplotypes were plotted onto a canonical variate analysis of shape. The resultant, apparently random, distribution of haplotypes throughout the morphospace defined by the first three canonical variates shows little association (Fig. 7). Nevertheless, discriminant analysis with cross validation shows that the best classification on the basis of shape variables is back to the parent haplotype group (Table 3) indicating some underlying association of shape and haplotype. However, AMOVA analysis (Table 4) suggests that neither shape groupings nor H and M are the covarying factor.

Variation was also high in the nDNA dataset (Table 2: G_{ST} = 0.1601, H_T = 0.3447, H_S = 0.2895). Mantel comparisons of the morphological Mahalanobis distance (D^2) with Nei's (unbiased) distance and the Prevosti distance from the nuclear-DNA RFLP data showed that, irrespective of the genetic distance matrix employed there is no evidence of association of shape and genotype; p [random z < obs. z] = 0.337 and 0.345 for association with the Nei and Prevosti distance respectively. However, as was found for the mtDNA dataset there is an association between nDNA genotype and shape grouping in that the allele CAL-2^A is found at the highest frequency in the south-central region where the haplotype ADA is encountered.

DISCUSSION

It is apparent that around the coastline of the UK and Ireland there is a separation of *L. saxatilis* into a thin-shelled, patulous, high-shore form (*L. saxatilis* H), and a thick-shelled mid-shore

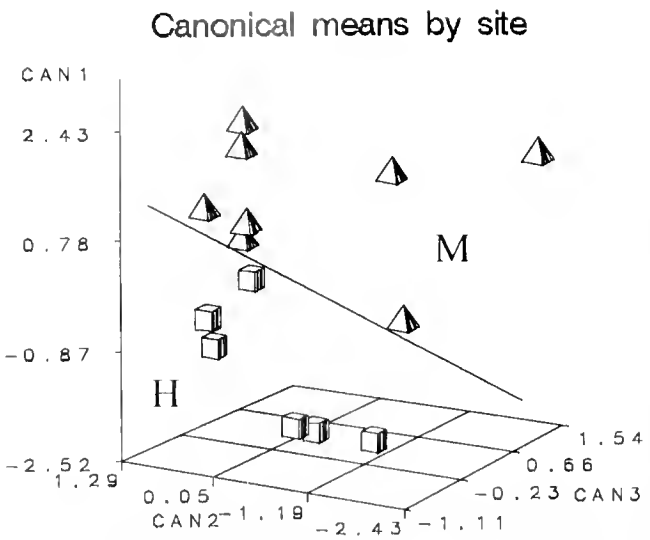

Figure 5. Population means from canonical variate analysis of south coast *L. saxatilis*.

form (*L. saxatilis* M). In this study we examined whether mitochondrial DNA variation provided evidence for genetic differentiation of these morphological varieties. At Galloway, Scotland, where repeated sampling on the shore has been undertaken, definite genetic differences are found between the morphs, with consistently different haplotype frequencies between H and M over both separate years and over multiple transects. Such repeatability shows the pattern is neither a temporal phenomenon, nor a simple distance effect. We found similar genetic differentiation between the Swedish E and S morphs of Janson (1982) which have similar morphologies to H and M. Interestingly, the differentiation was due to differing frequencies of the same haplotypes (high frequency of AAA in H/E and high frequency of BCA in M/S). Differentiation of mtDNA is also suggested at Mumbles (South Wales) where the frequency of AAA in *L. saxatilis* H is 0.3, compared to 0 in M — once again, it is the AAA haplotype which is involved. In contrast, no differentiation is indicated at Ravenscar or Ballynahown, but there is little mtDNA haplotype variation to partition at either of these sites.

On the south coast of England there are two obvious patterns to variation in *Littorina saxatilis* shell shape. On a broad scale, there are three "shape groupings" within which shape, analyzed by multivariate canonical variate analysis, is typically homogeneous but among which shape differs (Grahame & Mill 1992, Mill & Grahame 1995). Superimposed upon this, is the morphological distinction into H and M. Both mtDNA and nuclear DNA variation have been analyzed in samples from along the south coast to examine whether there is evidence that mtDNA differentiates H and M as at Galloway, and additionally if there is any evidence for restriction to gene flow between the shape groupings. Discriminant analysis of shape variation with mtDNA haplotypes as groups suggests a correlation between mtDNA haplotype and some aspect of shape on the south coast. However, there is no detectable association of mtDNA or nDNA with either shape groupings or H and M using AMOVA. Thus although a shape-mtDNA correlation has been detected it is not due to either of the *a priori* groupings considered here. In contrast to this, both an mtDNA haplotype and an nDNA allele at the CAL-2 locus are mainly limited to one of the shape groupings; the south central grouping of shape variation. It is likely that the AMOVA analysis of shape groupings does not detect this as a significant association due to the high variability of mtDNA within the shape groupings, effectively masking the 'between group' component. Nevertheless, the correlation of genetic differences at both the mitochondrial and nuclear DNA level with a known shape-group suggests that there is a real population difference.

Thus the pattern of genetic variation in *L. saxatilis* H and M is not simple. MtDNA evidence does not suggest that *L. saxatilis* H are consistently different from *L. saxatilis* M, although this can be the case on a particular shore. Given that these forms have different embryological characteristics (Hull et al. 1996) and display assortative mating (Hull 1998, Pickles and Grahame 1999) and that, in *Littorina*, there is a known substantial genetic component to shell shape (Grahame and Mill 1993), it is perhaps surprising

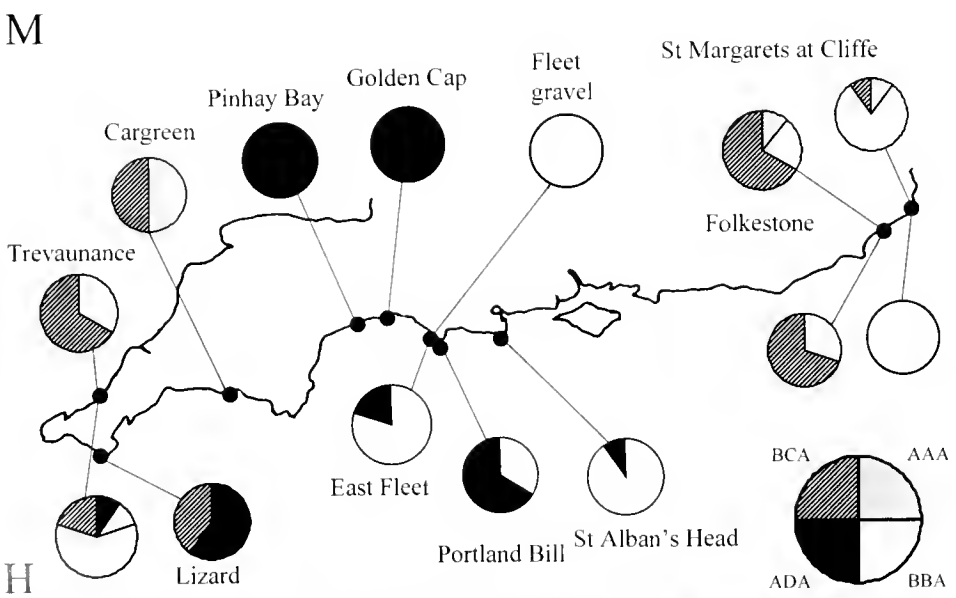

Figure 6. Distribution of the four mtDNA composite haplotypes along the south coast. *L. saxatilis* M are shown above the map and *L. saxatilis* H below.

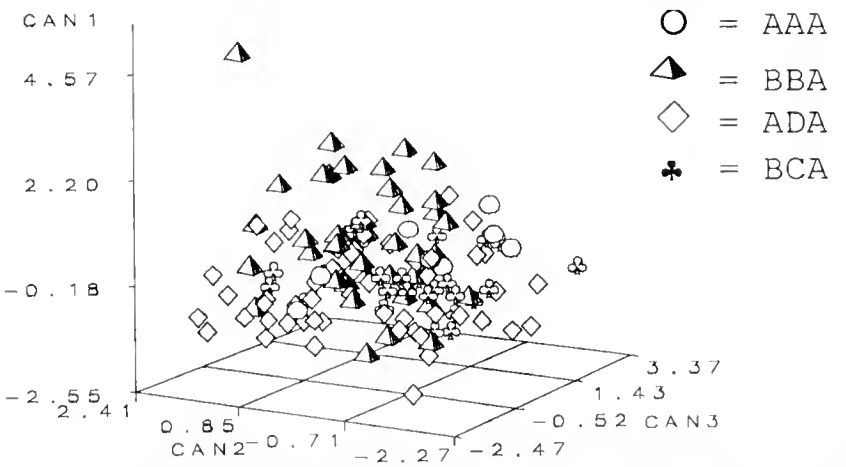

Figure 7. Plotting of haplotype onto canonical variate plot of shape measurements on an individual basis.

that clearer genetic differences have not been detected. However, the rough periwinkle group is itself young (Reid 1996) and sorting of mtDNA in recognized species has not gone to completion (Wilding et al. 2000a). Therefore the pattern within currently recognized species is likely to be complex, as noted here.

Is there a general framework for these observations? Apparently an aspect of the polymorphism of *L. saxatilis* (and perhaps of direct-developing intertidal snails in general) is the repeated appearance of similar phenotypes in different habitat regions of the shore, in response to similar selective pressures imposed by these habitats. Thus in the Galician region of Spain there are found a high-shore, ridged, banded and large morph (RB), and a low-shore, smooth, unbanded and small morph (SU) of *L. saxatilis*. These morphs show a variety of differences, considered to be at least partly genetically controlled, and are partially reproductively isolated (Johannesson et al. 1993). The likely selective factors are considered to be for small and slow growing snails in the lower shore, with larger and faster growing snails in the upper shore (Johannesson, Rolán-Alvarez and Erlandsson 1997). In the British Isles there are completely different morphs, referred to here as H and M, and it may be supposed that an important selective pressure is that of crab predation in the lower shore. Thus, *L. saxatilis* M closely resembles the common morph of *L. compressa* Jeffreys a low shore rough periwinkle, while *L. saxatilis* H is very like the typically higher shore *L. arcana* Hannaford Ellis. These H and M forms are like those in Sweden referred to as E and S (Janson 1982), but while in Britain there is evidence for partial reproductive isolation between H and M (Hull et al. 1996, Hull 1998, Pickles & Grahame 1999), this has not been reported previously for the Swedish animals (Erlandsson & Rolán-Alvarez 1998). Our observation of some difference in mtDNA haplotype frequency between them suggests that the E and S situation should be examined further.

The repeated nature of such phenotypic differences over large spatial scales (~1,000 km) together with the evidence of nascent reproductive barriers, yet superimposed on this undoubted evidence of gene flow between H and M (and E/S in Sweden, RB/SU in Spain), suggests analogy with the parallel speciation scenario proposed by Schluter and Nagel (1995) for sticklebacks. Here the proposed scale becomes important: it may be easier to envisage gene flow in *Littorina* populations along the British coast than between stickleback populations in isolated lakes, but gene flow between snail populations in Spain and Britain may well be very small. In addition the parallel speciation scenario may be applicable even over smaller scales (within Britain, within Galicia). Evidence to date from Galician *L. saxatilis* (Johannesson et al. 1983) and H and M around Britain for neutral loci (Wilding et al. in press) supports the interpretation that populations are more closely related at a site than between sites and yet display the same pattern of morphological differentiation.

The application of additional markers with replicated samples will aid in uncovering the genetic basis underlying *L. saxatilis* H and M. Further repeated sampling, as for that implemented at Galloway, is also needed to test if the patterns at Mumbles and Ursholmen, Sweden are robust to repeated sampling or simply an artifact brought about by the small sample sizes. It is clear that much more needs to be done on the biogoegraphy, behavior, and

TABLE 3.

Discriminant analysis with cross validation on canonical variance with haplotypes as predictors.

	Original haplotype group			
Destination haplotype group	**AAA**	**BBA**	**ADA**	**BCA**
AAA	62.50%	12.50%	12.50%	12.50%
BBA	11.70%	41.70%	23.30%	23.30%
ADA	22.00%	22.00%	41.50%	14.70%
BCA	38.50%	19.20%	25.90%	30.80%

TABLE 4.

Nested analysis of molecular variance (AMOVA) of RFLP variation in Littorina.

Source of variation	**% of total**
Group = shape zones	
among groups	6.78
among populations within groups	52.31
within populations	40.90
Group = 'H' and 'M'	
among groups	−6.70
among populations within groups	63.48
within populations	43.22

See Table 1 for details of placement of samples into particular groupings.

genetic constitution of the forms of *L. saxatilis* as an interesting example of speciation in progress.

ACKNOWLEDGMENTS

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ABSTRACTS OF TECHNICAL PAPERS

Presented at
The 21st Annual Meeting

MILFORD AQUACULTURE SEMINAR

Milford, Connecticut

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CONTENTS

OVERVIEW, 21ST MILFORD AQUACULTURE SEMINAR. **Walter J. Blogoslawski,** U.S. Department of Commerce, National Oceanic & Atmospheric Administration, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, 212 Rogers Ave., Milford, CT 06460

With over 170 registrants representing industry, research, and academic interests, the 21st Annual Milford Aquaculture Seminar had the highest attendance ever for this series of meetings.

This blend of both the theoretical and practical aspects of aquaculture ensured a meeting that permitted attendees to be exposed to areas of the practice of aquaculture outside their own expertise and provided a forum where the latest innovations were introduced and discussed.

The formal papers and posters were presented by persons from 10 U.S. coastal states and the countries of India and Canada. They represented members of three vocational aquaculture high schools, 14 universities, five marine labs, and several state and federal employees involved in shellfish and finfish aquaculture. Topics included aquaculture policy, crop insurance, education, disease, nutrition, and culture techniques. Studies provided information and experiences from laboratory research as well as full-scale commercial installations.

The Seminar has developed a tradition of offering the latest information available in the field in an informal atmosphere. This has succeeded in promoting a free exchange among all with an interest in the success and future of aquaculture. This Seminar continued that approach, allowing all attendees to enjoy and learn from the formal presentations, while also affording informal opportunities to discuss the latest developments pertinent to this important, expanding field.

The meeting was sponsored by the US Department of Commerce, NMFS Milford Laboratory, Milford, CT; abstract printing was courtesy of the US Department of Agriculture, Northeastern Regional Aquaculture Center, N. Dartmouth, MA. Their support is greatly appreciated.

WATER QUALITY AND NUTRITIONAL VALUE OF GREEN WATER VS. CLEARWATER CULTURING OF THE MARINE FISH, *TAUTOGOLABRUS ADSPERSUS*. **Bethann Balazsi,** Southampton College of Long Island University, Southampton, NY 11968, and **Gary H. Wikfors,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

Experiments were designed to test the relative importance of nutritional and water quality factors in green vs. clear water culturing of marine finfish larvae. Two microalgal strains, *Nannochloropsis sp.* (UTEX2341) and *Isochrysis sp.* (T-ISO), were used. Algae were added to fish tanks either in suspension or immobilized in beads or in a dialysis cassette. Two fish, *Tautogolabrus adspersus* (canners), were placed into each tank. Artemia was the food source. Artemia counts were done every day, as were per cent transmittance readings on algal samples from treatments with suspended algae. This was done to determine feeding rates of fish on artemia and of artemia on algae. Water samples were taken periodically throughout the experiment and analyzed for ammonia. The volume displacement was taken as the cunners were added to the tanks, and also at the termination of the experiment.

Immobilized algae in alginate beads was found to be unstable in seawater. Dialysis cassettes were found to be successful for eight days before the dialysis membranes lost integrity. In the dialysis cassette experiment, artemia were replenished 100% when resources were abundant. Algae were replaced each day. It was found that the artemia were cleared within 10 minutes to a few hours, depending on the treatment. The percentage of algae to replace declined in respect to the rate of clearing artemia. Volume displacement measurements did not show any significant growth of the cunner. Algae immobilized in dialysis cassettes removed ammonia from solution, but not as effectively as algae in free suspension. Nevertheless, these findings show dialysis cassettes are a useful tool in understanding how green water cultures work.

Funding provided by a Northeast Fisheries Science Center, Southampton College of Long Island University Cooperative Marine Education and Research (CMER) grant.

EFFECT OF SALINITY ON SURVIVAL AND GROWTH OF EARLY LIFE STAGES OF BLACK SEA BASS. **David Berlinsky, Rachel Howell, Jessica Henderson, Mark Watson** and **Terence Bradley,** University of Rhode Island, Fisheries, Animal, and Veterinary Science, Kingston, RI 02881

The black sea bass, *Centropristis striata*, is currently being investigated as a candidate for commercial aquaculture. One obstacle to production is high mortality rates during early life stages. Environmental salinity has been implicated as an important factor influencing larval survival in numerous species of finfish. The present study was conducted to determine the effects of a range of salinities on the growth and survival of black sea bass at several larval stages. Experiment 1 examined larval survival through yolk-sac absorption (3 days post hatch; dph) at salinities of 0, 5, 10, 15, 20, 25, 30 or 35 parts per thousand (ppt). Ten fertilized eggs were cultured in triplicate at each of the salinities in 500 ml glass containers. At 3 dph, the number of live larvae for each treatment was determined. In experiment 2, 380 actively feeding larvae (8 dph) were cultured in 40 L glass tanks at salinities of 15, 20, 25 or 30 ppt. Each treatment was replicated in triplicate. At 15 dph, the number of surviving larvae was enumerated for each treatment and a sample of 12 larvae from each tank was preserved in 10% neutral buffered formalin for measurement of length. In experiment 3, 200 actively feeding larvae (23 dph) were cultured in 40 L glass tanks at salinities of 15, 20, 25 or 30 ppt. Each treatment was replicated in triplicate. At 32 dph, the number of surviving individuals was enumerated for each treatment and a sample of 12 larvae from each

tank was preserved in 10% neutral buffered formalin for measure of length.

Results from experiment 1 indicate that salinities of at least 10 ppt are required for survival of black sea bass larvae. Survival improved from 42% at 10 ppt to 77% at 15 ppt to >90% at 20–35 ppt. In experiment 2, survival (67%) was higher at a salinity of 30 ppt than at 25 (41%), 20 (47%), or 15 (41%) ppt. No significant differences in growth were observed among treatments. In experiment 3, no significant differences in survival or growth of larvae were observed among the treatments. These studies indicate that black sea bass larvae require at least 10 ppt salinity and become more tolerant to lower environmental salinities with development.

A THEORETICAL ESTIMATE OF THE POTENTIAL IMPACT OF ASIAN SHORE CRAB PREDATION ON MUSSEL SETTLEMENT. Diane J. Brousseau and **Amy Filipowiez,** Fairfield University, Biology Department, Fairfield, CT 06430

The Asian shore crab, *Hemigrapsus sanguineus*, readily consumes juvenile blue mussels, *Mytilus edulis*. In laboratory feeding experiments (n = 59; sexes pooled) each *Hemigrapsus* ate an average of 12.7 ± 11.6 mussels (<10 mm SL) per day over a period of three days. Using this daily consumption rate along with published estimates of mussel recruitment rates in the field (Chipperfield, 1953; Menge, 1978; Petraitis, 1990; Petraitis, 1991) and crab densities at intertidal sites in Long Island Sound (Ahl & Moss, 1999; Gerard et al., 1999), theoretical estimates of mortality due to *Hemigrapsus* predation were calculated. Estimates of mortality ranged from 28–115% of annual mussel settlement, depending on recruitment strength. The average estimate of mortality for all recruitment rates considered (n = 7) was 64%. Under conditions where field consumption rates are comparable to those measured in the laboratory, *Hemigrapsus* has the potential to seriously limit mussel populations in areas where the two species co-occur.

PRELIMINARY INVESTIGATIONS OF CRAB PREDATION ON BAY SCALLOPS. Joseph Choromanski and **Sheila Stiles,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

In the course of bay scallop aquaculture research conducted at the National Marine Fisheries Service Laboratory in Milford, CT, excess scallops were donated to Connecticut municipal shellfish commissions for free-planting in area waters. The practice of free-planting, or tossing seed scallops (10–40 mm) directly into the water, has come under scrutiny because of the observable decreasing return in the number of adults (>60 mm) caught by recreational fishermen in the towns that have such policies. Field studies of bay scallops have suggested a variety of causes for population fluctuations including habitat loss, genetic inbreeding depression, and predation. It is generally known that crab predation can be a major factor in survival and growth of bay scallops for reseeding or stock enhancement efforts, especially in sites devoid of eel grass, which can serve as a refuge for small scallops.

To evaluate crab predation on scallops, an experimental study was conducted with green crabs (*Carcinus maenas*) in the laboratory. Six treatment aquaria with 10°C flowing seawater were established with a single crab and 10 scallops. A seventh aquarium, with 10 scallops and no crab to check for non-predator related mortality, was used as a control. Four separate trials were run with scallops in each of the following size classes: 10, 20, 30 and 40 mm shell height. Trials were duplicated with a second set of crabs and scallops. Observations were made at 1, 6, 20, and 48 hours for each study. Results indicated that larger-sized scallops had higher survival rates, indicating some degree of refuge from predation by green crabs. In addition, damage to scallop shells was manifested in a characteristic appearance which could be used in identifying mortality by crab predation in the field.

A smaller scale project involved a comparison of bay scallop predation by Asian shore crabs (*Hemigrapsus sanguineus*) versus similar-sized green crabs. Six containers were set up for each crab species with 15 scallops and one crab per container. The shell height of scallops ranged from 6–8 mm and the carapace width of crabs ranged from 18.0 to 25.3 mm. Observations were made after 48 hours when the experiment was terminated. The most notable difference was the number of scallops eaten by the male and female crabs. Generally, male crabs ate all of the scallops in their containers, while the female crabs ate very few to none. This difference could be attributed to the larger size of the claw of the male crabs. There was a slight difference in the number of scallops eaten by the green crabs versus Asian shore crabs. These observations can be used for planning purposes when attempting to enhance or replenish scallop populations.

REPORT ON DESIGN OF PHOTOVOLTAIC-POWERED FLOATING UPWELLER SYSTEM FOR GROWTH OF *CRASSOSTREA VIRGINICA* AND *MERCENARIA MERCENARIA*. Todd Corayer, Block Island Shellfish Farm, Ocean Ave., P.O. Box 1342, Block Island, RI 02807

The intent of this experiment was to design, construct, and evaluate the performance of a floating shellfish upweller which would offer the same growth enhancement opportunities of a traditionally powered unit, yet be completely independent of shoreside power. The unit would be adaptable to salt pond shellfish farms where traditional power was unavailable or cost prohibitive.

The concept and construction was partially funded through an R&D Partnership Award from the Slater Center for Ocean Technology at the University of Rhode Island and through an alternative energy grant from the United States Department of Energy. Block Island Shellfish Farm (BISF) formed a partnership with Chris Warfel PE, owner of Enteeh Engineering, specialists in solar

and wind energy systems, to facilitate the electrical engineering of the upweller.

The upweller consists of single and double-tiered PVC silos with submersible pumps positioned atop. The shellfish were divided between the silos with a test case being established without the aid of a water pump to identify any ancillary growth assistance from the structural design of the upweller. Another test case was located in the abutting lease of Block Island Shellfish Farm (BISF). These test case animals were placed in 1 mm ADPI grow-out bags and maintained along with BISF's inventory of *Crassostrea virginica* and *Mercenaria mercenaria*. Animals in both sites were measured for volume and size changes regularly, and collected data were evaluated against the traditional farm-raised test cage.

Provisions were incorporated into the design to prevent the silos from coming into contact with the pond bottom in situations of extreme low water events. Constructed of pressure treated lumber, the unit was also designed to survive any severe weather which a coastal pond might endure. An insignificant amount of heavy metals leaching was detected as a result of systematic water testing inside the upweller as well as up and down stream.

At this point in our research, regular measurement recordings indicate a successful mating of the photovoltaic cells and the specially designed upweller silos. Essential to the success were animal-to-screen densities and the ability to regulate water flow rates. Experiments are ongoing to refine the structural elements of the unit and the silos to decrease maintenance requirements.

DEVELOPMENT OF THE DIGESTIVE CAECUM DURING THE FIRST LARVAL STAGE IN THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS*. **Yvonne Coursey** and **Mary Kimble**, Department of Biology, University of South Florida, Tampa, FL 33620

The internal development of *Limulus polyphemus* is not complete at the time of hatching from their embryonic membranes. A 1st instar, an animal that has just hatched from its egg casing, does not have a complete digestive system. It lacks the digestive cecum, which branches off from the intestine, and thus can't absorb nutrients. Instead, the 1st instar relies on the yolk present in compartmented areas where the gut will arise. The foregut, which includes the esophagus, crop, and gizzard, is fully formed in the 1st instar. In addition, the hindgut is also present. The intestine and the digestive cecum develop as the animal progresses from a 1st to a 2nd instar, approximately 11–14 days. In this time period the midgut and digestive cecum develop, becoming contiguous with the foregut and the hindgut.

Juvenile horseshoe crabs, 1st and 2nd instars (a 2nd instar is a 1st instar that has molted.), were fixed in Bouin's fluid or a 3% formaldehyde/seawater solution. They were dehydrated through an alcohol gradient series, and embedded in Unicryl® or Spurr's resin. Sections were cut at 3–4μm for light microscopy and 60–90 nm for electron microscopy.

This study determined the internal morphology of 1st and 2nd instars as a precursor to the identification of the site(s) of hemopoeisis in *Limulus polyphemus*. Horseshoe crab blood cells are used to produce *Limulus* amebocyte lysate (LAL), which is used to test for sterility of intravenous solutions. A long-term goal of our research is to establish methods for culturing horseshoe crab cells *in vitro*.

HORSESHOE CRAB AQUACULTURE: RESULTS FROM INITIAL SPAWNING STUDIES. **Carmela Cuomo**, Yale University, Department of Geology & Geophysics, New Haven, CT 06520, and **Paul R. Bartholomew**, SUPERB Technical & Environmental, Hamden, CT 06517

The horseshoe crab, *Limulus polyphemus*, is considered by many to be a living fossil, more for its primitive appearance than for its actual ancestry. The actual species dates back only about 20 million years, although its relatives can be traced back over 200 million years in the fossil record. At the present moment in time it is, perhaps, more important to the biological community than to the geological one. Sea turtles and at least 20 species of Arctic-bound shorebirds feed upon *Limulus* eggs deposited in the shallow tidal flats all along the eastern seaboard of the United States during their spring migrations. Several critical "staging areas" for these birds, including Milford Point, CT, are also known to be "critical habitats" for *Limulus* spawning. Furthermore, *Limulus* adults are harvested for "bleeding" by several biomedical companies. *Limulus* blood is a copper-based blood that coagulates in the presence of gram-negative bacteria. Several companies have developed a biomedical assay, known as *Limulus* amoebocyte lysate (LAL), which is used to test for human diseases (e.g. spinal meningitis and gonorrhea), the purity of pharmaceutical products, and clinical laboratory water purity. The demand for LAL doubled between the 1980s and the 1990s, and doubled again between 1990 and 2000. It is predicted to keep rising well into the 21st century.

Coincident with the increased demand for *Limulus* has been a drastic decline in the *Limulus* populations along the central and northeastern Atlantic Coast of the United States. This decline has been attributed to many factors including over-fishing, declining spawning grounds, deliberate destruction, increased harvesting for LAL, and pollution. In 1998, the Atlantic Marine Fisheries Commission prepared a report on the status of the horseshoe crab populations along the east coast of the United States and a management plan for the fishery. The central goal of this plan was to conserve and protect the horseshoe crab resource in order to maintain sustainable levels of standing stock to ensure both its important role in natural ecosystems, as well as its role in commercial enterprises. Although this is a good initial step, it does not represent the solution to the declining horseshoe crab populations. Breeding grounds are likely to continue to decline due to increased

shoreline development, increased biomedical demands on horseshoe crab, and inability to maintain a steady-state. More fishermen are turning to commercial eel and conch fishing as other fishing stocks decline. The solution to the problem lies in the ability to produce a reliable stock that can be maintained even as natural stocks undergo population fluctuations.

To this end, in the summers of 1999 and 2000, preliminary studies were undertaken at the NMFS laboratory in Milford, CT to test whether or not it was possible to induce *Limulus* adults to spawn in captivity. Various factors were tested for their importance, including sediment grain size, tidal height, light, and tidal regime. The results from this research have proved promising — 4 pairs of adult *Limulus* successfully mated, resulting in the production of thousands of eggs. The eggs were allowed to develop under normal conditions and had a hatching rate of approximately 60% over a two month period. Experiments evaluating the long-term survival and growth of the larvae are presently underway. It is our goal not only to rear the eggs successfully through hatching, but also to rear the hatchlings successfully through at least the first seven molts. It is anticipated that the results from these experiments will translate directly to industry as they will provide horseshoe crabs to companies that need them for years to come.

A COOPERATIVE STUDY ON THE CULTURE OF *CHONDRUS CRISPUS* (RHODOPHYTA) FOR POSSIBLE COMMERCIAL AND BIOREMEDIATION APPLICATIONS IN LONG ISLAND SOUND. John J. Curtis, Sherry W. Lonergan and **Paul J. Trupp,** Bridgeport Regional Vocational Aquaculture School, 60 St. Stephens Rd., Bridgeport, CT 06605; **Jose Zertuche,** University of Connecticut, Stamford, CT 06901, and Universidad Autonoma De Baja California, Ensenada, B.C., Mexico; **Rachel Carmona** and **Charles Yarish,** University of Connecticut, Stamford, CT 06901

As part of an agreement with the University of Connecticut at Stamford, students and staff at the Bridgeport Regional Vocational Aquaculture School are participating in a cooperative study on the feasibility of growing *Chondrus crispus* (Irish Moss) in Long Island Sound for commercial purposes, and as a possible candidate for bioremediation. Dr. C. Yarish, Professor of Ecology and Evolutionary Biology, and Dr. J. Zertuche, a visiting scientist and Professor from Universidad Autonoma De Baja California, formally introduced *Chondrus crispus* culture techniques used on Prince Edward Island, Canada and Baja, California to our school as a project proposal. Since the initial introduction, these scientists and other UConn faculty and staff have visited our school to teach the students the life history and biology of *Chondrus*, and also the design and construction of the equipment necessary for its experimental commercial culture

The first task was to assemble the components necessary to maintain and nurture the *Chondrus* in the laboratory setting. An available .61 × 1.20 × 2.44 m (height × width × length) gel-coated fiberglass tank with a gray interior was selected. To provide aeration and the required water turbulence, a 1 horsepower regenerative blower was used to inject air, through the use of a manifold, into four (4) 1.27 cm PVC pipes. Each 1.27 cm pipe was drilled, in a single horizontal row, with 1.6 mm diameter holes spaced 2.54 cm apart. The drilled pipes were placed in the bottom of the tank with the holes facing upward and running parallel to the 2.44 m of the tank. They are equally spaced from each other and leveled to provide for an equal amount of air discharge for a consistent tumbling action. Suspended above the tank is a 1000-watt metal-halide light fixture delivering 400 μ mol photons m^{-2} s^{-1}, which is on a timed cycle 10:14, L:D cycle. The water is obtained from Long Island Sound and maintained in the tank at a salinity of 27 ppt and at 15 C through the use of a chiller. The water depth is maintained at .36 m to allow for the correct tumbling action and with consistent tissue-to-water volume ratio (<1g /1 L).

The first batch of *Chondrus* was collected by Dr. Zertuche and UConn staff at a site in Stamford, CT and placed in the prepared tank at The School. Students then sorted the *Chondrus* into viable and non-viable plants. Plants with too many (over 50% coverage) epiphytes and epifauna (i.e. oyster drills, star tunicates, snails, and bryozoans), were considered not viable and returned to Black Rock Harbor. The *Chondrus* that was viable was cleaned of as many epiphytes and epifauna as possible and placed in the prepared tanks in the lab. The *Chondrus* was fed twice a week using a 10:1 nitrogen-to-phosphorus ratio. Twenty-four hours after feeding the *Chondrus* in the tank, the tank was drained to remove excess nutrients, cleaned, and refilled with new seawater.

The next task was to construct the equipment that is used to load and unload the *Chondrus* into mesh "stockings" (mussel socks) for outdoor culture. The apparatus was built identical to those used for experimental commercial culture in Mexico. Dr. Zertuche and UCONN staff demonstrated to the students the proper method of loading and unloading the socks. They emphasized the economic importance of a fast and an efficient system to minimize operating costs.

After two weeks in the growout tank, students sorted the *Chondrus* according to size. The larger specimens were used for the outdoor growout segment of our experiment and the smaller sized material was retained in the growout tank. A two-meter square frame, made from 7.62 cm PVC pipe, was constructed to hold the mussel socks at a 0.20 m depth in the water column. Five mussel socks were filled with the *Chondrus* and secured to the raft with wooden button-type fasteners. The raft was deployed in Black Rock Harbor in Bridgeport, CT on December 20, 2000. The *Chondrus* will be weighed to determine the growth, internal levels of carbon, nitrogen, and phosphorus, and water quality parameters as well every four weeks in the winter. In the spring, the interval will be reduced to every two to three weeks to determine the aforementioned parameters as a function of season.

THE EFFECTS OF STOCKING DENSITY IN PEARL NETS ON THE SURVIVAL, GROWTH, AND REPRODUCTIVE POTENTIAL OF THE BAY SCALLOP, *ARGOPECTEN IRRADIANS IRRADIANS*. **Maureen Davidson,** New York State Department of Environmental Conservation, 205 North Belle Mead Road, East Setauket, NY 11733

The stocking densities under which bay scallops (*Argopecten irradians irradians*) are reared may affect survival, growth, and reproductive potential. In order to investigate the influence of stocking density on scallop production, hatchery-reared bay scallops were held in pearl nets at three densities, 80/pearl net, 240/per net and 800/per net for 67 days during the summer. Surviving scallops were counted and shell heights were measured to determine growth. The animals were transferred to lantern nets and stocked at two different densities, 50/tier and 200/tier, and overwintered, grouped by their initial densities in the pearl nets. The following spring survival, shell heights, and gonadal index (a measure of reproductive potential) were determined.

Survival and growth were inversely proportional to stocking density in both pearl and lantern nets. Stocking density in the lantern nets was found to have a greater effect on overwintering survival than did the density in pearl nets. Gonadal indices indicated that scallops initiated spawning at the same time, regardless of density treatment. Once spawning began, bay scallops held at 50/tier in the lantern nets maintained greater gonadal indices and for a longer period of time than those held at 200/tier. There was no significant pearl net effect observed on gonadal index. Bay scallops cultured for direct market should be held at low densities in pearl and lantern nets to maximize growth and survival. Bay scallops raised for resource restoration should be held at moderate densities in pearl nets and low densities in lantern nets to maximize survival and reproductive potential.

DESIGN AND OPERATION OF AN AIRLIFT DRIVEN FLOATING UPWELLER SYSTEM. **Christopher V. Davis,** Pemaquid Oyster Company, P. O. Box 302, Waldoboro, ME 04572

Aquaculturists often employ various types of upwelling systems to expedite the nursery phase of bivalve culture. Land-based systems provide for ease of maintenance and security, but incur high pumping costs. In contrast, Floating Upweller Systems (FLUPSY's) greatly reduce or eliminate pumping costs due to the minimal hydrostatic head required to drive water past the juvenile shellfish. FLUPSY's typically employ centrifugal or axial flow electric pumps to propel water through the system. In contrast, low head airlift pumps are capable of moving large volumes of water, are inherently simple to construct and maintain, eliminate the risk of electrical shock, and have lower operating costs compared to most electrically driven water pumps. Pumping efficiency in airlift systems will be discussed as it relates to the air supply, depth of air injection, lift, diameter of the eduction pipe, and air flow. The design and operation of an airlift-powered FLUPSY operated in Maine will be described.

GROWTH AND MORTALITY OF THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA*) AND THE QUAHOG *(MERCENARIA MERCENARIA VAR. NOTATA)* IN A TAYLOR FLOAT IN MILFORD HARBOR. **Jasmine DeCrescenzo,** Nonnewaug Ellis Clark Regional Ag-Science Technology School, Supervised Agriculture Experience Program, 5 Minor Town Road, Woodbury, CT 06798; **Joseph DeCrescenzo** and **Inke Sunila,** State of Connecticut, Department of Agriculture, Bureau of Aquaculture, P.O. Box 97, Milford, CT 06460

Because of the devastating effects of disease on the natural oyster and clam populations in the Northeast, there has been a growing interest for using hatchery-raised seed. Connecticut receives most of its hatchery-raised stock from neighboring states. The object of this project was to evaluate growth and mortalities of oysters and hard clams during their nursery stage. The shellfish were grown in Milford Harbor, Connecticut during the summer and early autumn of 2000, using the Taylor Float suspended culture system.

Four thousand eastern oysters, *Crassostrea virginica,* (4.5 mm in size) and seven thousand quahogs, *Mercenaria mercenaria* var. *notata,*(4 mm in size) were received from two hatcheries in New York. Both the oysters and clams were divided into two groups, a and b, and placed into a Taylor Float. The float was then placed at the end of a dock in Milford Harbor, which represents a typical estuary that could be used for an aquaculture endeavor. Bivalves were checked on a bi-weekly basis for growth and mortality. The temperature ranged from 15°C to 25.2°C and the salinity from 15.4‰ to 26‰, during the project. Data were collected over a 13 wk period from July into October. Oysters from both groups a and b showed significant growth throughout the project, reaching an average of 37 mm in length at the last measurement. No mortalities were recorded for the oysters. The hard clams grew to 9 mm in length with 19% cumulative mortality at the experiment's conclusion.

From the data collected, oysters showed substantially greater growth in comparison to the clams in the suspended culture. In conclusion, oysters would be a better species of bivalves for growing in the Taylor Float system in the tested estuarine environment. *Mercenaria mercenaria* var. *notata* may be better adapted for growth in full-strength ocean water.

ROTIFER PRODUCTION ON MICROALGAL DIETS: FIRST STEPS TOWARD PROCESS ENGINEERING. **Mark S. Dixon** and **Gary H. Wikfors,** USDOC, NOAA, National MarineFisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; and **Bethann Balazsi,** Long Island University, Southampton College, Natural Science Division, Southampton, NY 11968

Live food production is a critical component in the successful culturing of marine finfish. Consistent production of microalgal biomass, and the efficient conversion of that biomass into live food, leads to reliable nutrition of cultured fish. A series of small-scale experiments was conducted to optimize microalgal diets, define rearing conditions, and explore the potential for process control of production for rotifers (*Brachionus plicatilis*).

The microalgal strain PLY429, *Tetraselmis chui*, yielded the greatest rotifer production when compared to several commonly used strains. A constant density of 6 million microalgal cells per millilter and initial low rotifer stocking densities resulted in rapid reproduction and high overall production. Under these conditions, rotifer populations doubled in as little as two days and reached a maximum density of over 2,000 per milliter in 6 days.

A spectrophotometer was used to monitor algal densities, algal cells were added manually to maintain the target density, and rotifers were counted manually. There is good potential to automate the entire process using "off the shelf" technology. This potential will be explored in upcoming experiments.

DEVELOPMENT OF ASSAYS TO EVALUATE IMMUNE FUNCTIONS OF AMERICAN LOBSTERS (*HOMARUS AMERICANUS*) AND THEIR USE IN FIELD STUDIES. **Shuyun Dong** and **Sylvain De Guise,** University of Connecticut, Department of Pathobiology, Storrs, CT, 06269

In order to develop tools for monitoring heath of American lobsters, we developed assays to evaluate lobster immune functions and validated them in field studies (an EPA study on contamination of lobsters around dredge-material dumpsites and a UConn study monitoring Long Island Sound lobster health).

Hemolymph of lobsters was aspirated and mixed with acid-citrate-dextrose as anticoagulant. Hemolymph appearance was described according to the color of the fluid and hemocytes were counted with a hemocytometer. Phagocytosis of hemocytes was determined by flow cytometry. A cell culture system maintaining lobster hemocytes *in vitro* was established.

Our field study results showed that although hemolymph of lobsters varied from gray to greenish or orange, there was no statistical difference between the different locations. Phagocytosis of lobsters from one location was statistically higher than that from any other locations, and interestingly, hemocyte counts of lobsters from this location were significantly lower than 4 out of the 7 other groups. Even though phagocytic index using two different parameters correlated very well ($r=0.82$), there was no correlation between phagocytosis, hemocyte counts, and hemolymph appearance.

Our lab work demonstrated that sea water with 10% FCS at 12°C represented the best *in vitro* cell culture conditions for lobster hemocytes. Cells adhered to the surface of the 24-well plate and adopted amoeboid shape. Some cells were elongated or fusiform and had prominent filamentous or round pseudopodia. According to cell size and cytoplasm granule, the cells might be differentiated into at least 2 types: hyaline hemocytes and granulocytes. Live cells were found for as long as 48 hours of incubation. Results of analytical toxicology and histophathology will allow the final validation of our field results on the significance of immune function assays. The correlation between immunology and toxicology will provide useful information for the evaluation of lobster health. Our future work will focus on developing other immune function assays such as respiratory burst, NK cell-like activity, hemocyte proliferation, and apoptosis, in addition to experimental immunotoxicology. We expect that those studies will be helpful in understanding the health status of lobsters and identifying the cause(s) and contributing factors involved in the recent Long Island Sound lobster die off.

IMPORTATION OF CHINESE CLAMS CAUSES PROBLEMS IN LOCAL MARKETS. **Gef Flimlin,** Rutgers Cooperative Extension, 1623 Whitesville Rd., Toms River, NJ 08755

In early 2001, there was an importation of frozen molluscan shellfish (clams & mussels) into New Jersey originating from the Republic of China. The state embargoed almost 6,000 cases of clams both whole and half shell which were falsely labeled as cooked or pre-cooked and were found to be raw. Once the State of New Jersey and the US Food and Drug Administration (FDA) investigation revealed that these were not, in fact, cooked clams, the destruction of the products and a national voluntary recall was initiated. The FDA labs later isolated Hepatitis A virus in samples from the shipment implicated in an outbreak.

There is a concern that raw or partially cooked molluscan shellfish is entering the United States under false pretenses as a cooked product. This appears to be a circumvention of the Memorandum of Understanding (MOU) process for a foreign country to be evaluated under the criteria of the National Shellfish Sanitation Program (NSSP) prior to shipping products into the U.S. At the heart of the NSSP is the knowledge that states and countries with FDA MOU's have a program which complies with certain critical water quality criteria including bacteriological, chemical, and marine biotoxin hazards relative to the shellfish and the growing waters. Hazards such as chemicals and marine biotoxins obviously can not be destroyed via cooking.

With the increase of world trade and shellfish aquaculture, it is imperative that the domestic shellfish industry be aware of imported molluscan shellfish and advise the FDA and state depart-

ments of health to force foreign countries to adhere completely to the NSSP Memorandum.

COMPARATIVE EFFICIENCY OF HORMONES ON THE MATURATION AND BREEDING OF INDIAN CATFISH, *HETEROPNEUSTES FOSSILIS*. **Thommai A. Francis,** Department of Fish Farm Management, Fisheries College and Research Institute, Tuticorin, India.

Recent reports show that the ovulation of this species can be induced even during non-spawning season not only through environmental manipulation but also by hormonal stimulation. Various hormones (i.e. carp pituitary extract, human chorionic gonadotropin, mixture of HCG & pituitary extract, leutinizing hormone releasing hormone (LHRH), and ovaprim) were used in the present work to study the maturity and ovulation in *Heteropneustes fossilis*. Among the different hormones, pituitary extract showed higher Gonadosomatic index (GSI) in the injected fish followed by human chorionic gonadotropin. Natural hormones (pituitary extract and human chorionic gonadotropin) influenced the maturity of *Heteropneustes fossilis* better than synthetic hormone analogs.

Among the different hormones used for induced breeding of *Heteropneustes fossilis* better results were obtained from ovaprim. The rate of fertilization (80–84%) was higher in ovaprim-induced fish, while in the case of the other hormones it was less than 78%. Changes in spermatozoa count and the biochemical composition of milt of *Heteropneustes fossilis* were studied by injection of different hormones. Maximum spermatozoa count (3.67×10^6) was recorded in the case of human chorionic gonadotropin followed by the mixture of pituitary and HCG-injected fish (3.21×10^6) and pituitary extract (3.17×10^6). Glucose content of milt was maximum (0.07 mg/0.05 mℓ) in human chorionic gonadotropin-injected fish. Maximum protein content (0.06 mg/0.1 mℓ) was observed from LHRH-induced fish. Data obtained in the experiments were statistically analyzed by analysis of variance.

CHARACTERIZATION OF *VIBRIO CARCHARIAE* AS A PATHOGEN OF SUMMER FLOUNDER (*PARALICHTHYS DENTATUS*). **Eric Gauger** and **Marta Gómez-Chiarri,** University of Rhode Island, Fisheries, Animal and Veterinary Science, Kingston, RI 02881

Vibrio carchariae is a recently identified bacterial pathogen of summer flounder (*Paralichthys dentatus*). As such, little is known about the etiology of the disease which has been given the name Flounder Infectious Necrotizing Enteritis (FINE). Preliminary work on the disease focused on identification of the pathogen, description of clinical and histological symptoms of the disease and determination of a LD 50 at one temperature using intraperitoneal (IP) injection. Current research is focusing on the route of infection and the effects of environmental and other parameters on the virulence of the pathogen.

Disease challenges were conducted at 20, 22 and 24°C, using IP injection. No significant differences in the LD 50 value were seen over this temperature range. Potential routes of infection are being investigated through a set of controlled disease challenges. Groups of fish were subjected to bacterial inoculations approximately 100 times the LD 50 dose for IP injection. Intramuscular injection caused mortalities. However, the clinical signs did not match those of FINE. Immersion was able to cause some infections, but they were limited to surface lesions and did not cause mortalities. Both gastric and intestinal intubation initially failed to produce disease, suggesting that the bacteria may not be able to survive the passage through the digestive system.

A second experiment was conducted at higher stocking densities, immediately after transporting the fish from the main flounder facility at URI. In this experiment, gastric intubation was able to cause mortalities. These results suggest that some sort of stress may be required for the disease to develop. Future work will attempt to clarify the link between stress and disease, investigate the effect of stocking density and water quality on pathogenicity, and also determine other potential routes of infection.

FLOW CYTOMETRY AS A TOOL TO QUANTIFY OYSTER DEFENSE MECHANISMS. **Michael J. Goedken** and **Sylvain De Guise,** Department of Pathobiology, University of Connecticut, Storrs, CT, 06269

The fast-growing oyster aquaculture industry is greatly hindered by two parasites (MSX and Dermo), which can kill up to 80% of the harvest. The relationship between parasites and oyster defense mechanisms is unclear. Two defense mechanisms of the Eastern Oyster (*Crassostrea virginica*) were quantified at the single cell level utilizing flow cytometry. Phagocytosis was measured using fluorescent beads. Respiratory burst activity was quantified as the increase in dichlorofluorescein-associated fluorescence upon stimulation.

These two assays distinguished three populations of hemocytes (granulocytes, hyalinocytes, and intermediate cells) with unique functional characteristics. Granulocytes were most active at phagocytosis and peroxide production while hyalinocytes were relatively inactive. The intermediate cells had moderate phagocytic and respiratory burst activity. Flow cytometry can rapidly, accurately, and directly quantify the morphology and function of a large number of individual cells, and will lead to a better understanding of the yet enigmatic bivalve immune system.

COMPARISON OF TWO TRAP TYPES USED IN A MUNICPAL GREEN CRAB PREDATOR CONTROL PROGRAM. **David W. Grunden,** Shellfish Constable, Town of Oak Bluffs, P. O. Box 1327, Oak Bluffs, MA 02557

This study was conducted in Sengekontacket Pond, a 716-acre salt pond on Martha's Vineyard Island in Oak Bluffs, Massachusetts. Funding was provided through a grant from the Southeastern

Massachusetts Aquaculture Center (SEMAC). The efficiency of two types of traps in catching green crabs (*Carcinus meanus*) was evaluated.

One trap was the traditional eel pot that has been used in the Oak Bluffs Shellfish Department predator control program for the past 15 years. The eel pot is constructed of half-inch mesh vinyl coated wire.

The other trap was a small fish trap manufactured by the Fukui Company. This trap is constructed from a heavy vinyl coated wire frame with half-inch mesh plastic netting.

The traps were baited the same, using fish scraps and heads donated by "Net Result", a local fish market. The number of traps tended and number of bushels caught in each type of trap was recorded each time the traps were tended. From these data, a catch per unit effort was calculated.

The results clearly indicate that the Fukui fish trap is more efficient. Also, despite the higher initial cost of the Fukui trap, it is the more economical trap to use based on the greater trapping efficiency of 2.6 times the traditional eel pot.

EFFECTS OF PHOTOPERIOD MANIPULATION ON REPRODUCTION IN THE BLACK SEA BASS, *CENTROPRISTIS STRIATA*. **Rachel Howell, David Berlinsky,** and **Terence Bradley,** University of Rhode Island, Fisheries, Animal, and Veterinary Science, Kingston, RI 02881

The black sea bass, *Centropristis striata*, is currently being investigated as a potential candidate for commercial aquaculture. As with many new species, one of the most significant constraints limiting commercial production of black sea bass is the lack of a reliable supply of eggs and larvae. A narrow window of annual spawning prevents year-round availability of juveniles for growout and *C. striata* fail to mature and spawn in captivity without hormonal induction. Given that the ability to control the reproductive cycle and the establishment of reliable spawning methods are critical for enhancing production of this species, the primary objective of the present study was to investigate whether black sea bass could be induced to spawn out of season using photoperiod manipulation.

Adult black sea bass were reared under a simulated natural or 6 mo compressed photoperiod regimen. Both groups began the experiment at a minimum day length of 9L:15D and a minimum water temperature of 15°C. Water temperature was increased seasonally with photoperiod and maintained at 18°C once maximum day length was reached (15L:9D). Reproductive development in these fish was monitored by gonadal biopsies and by assay of plasma levels of gonadal steroids (17β-estradiol, testosterone, and 11-ketotestosterone). At monthly intervals, fish were weighed, measured, and bled, and gonadal tissue sampled by biopsy. Following histological processing, biopsy samples were examined to determine the stage of oocyte maturation and maximum follicle diameter. Females with follicles ≥500 μm in diameter were induced to spawn by implantation of 25–50 μg leutinizing hormone-releasing hormone analog ($LHRH_a$). Females reared under the compressed cycle attained follicles 500 μm in diameter and were induced to spawn in March, approximately 2 mo in advance of individuals reared under the simulated natural photoperiod. Males on both cycles began spermiating in late January and continued through the duration of the experiment. Measurement of plasma levels of 17β-estradiol, testosterone, and 11-ketotestosterone are ongoing and will be presented.

AQUA-TOURISM, TURBO-TIDAL UPWELLERS, AND OTHER DEVELOPMENTS IN THE SHELLFISH INDUSTRY ON MARTHA'S VINEYARD IN THE YEAR 2000. **Richard C. Karney,** Martha's Vineyard Shellfish Group, Inc., Box 1552, Oak Bluffs, MA 02557; **John C. Blake,** Sweet Neck Farm, Box 1468, Edgartown, MA 02539; and **Thomas E. Berry,** Martha's Vineyard Shellfish, Box 1660, Edgartown, MA 02539

When polled, shellfish growers in Massachusetts have consistently identified the general public's perception of aquaculture as one of the most important factors affecting the growth of aquaculture in the Bay State. The strong and enduring tradition of active government participation by the Massachusetts citizenry makes a public education project which stresses the benefits of shellfish aquaculture crucial to the development of an industry that relies on the use of the marine common lands. Under grant funding from the Massachusetts Department of Food and Agriculture, the Martha's Vineyard Shellfish Group conducted a collaborative, multifaceted public relations project with the goals of showcasing local public and private aquaculture operations and extolling the environmental, economic, and social benefits of the shellfish aquaculture industry. We employed a multitude of communication outlets to spread the gospel of aquaculture. The project included aqua-tours of private shellfish farms, web site development, tastings of cultured shellfish, public access cable programs, posters, and the distribution of printed information.

With financial support from the National Fish and Wildlife Foundation, five growers cultured over a million seed oysters in tidal upweller nurseries. Some of the nurseries were modified with the addition of side vents. Although these "turbo-charged" nurseries experienced an improved flow rate, there appeared to be no improvement in oyster growth rate compared to nurseries without the added vents.

Both public and private oyster stocks were tested for disease. Private growers in Katama Bay were relieved to find that their cultured oysters, which were believed to be infected with MSX (*Haplosporidium nelsoni*) upon PCR analysis, were found to be infected with the less serious SSO (*Haplosporidium costale*). Oysters sampled for Dermo (*Perkinsus marinus*) from public beds in Tisbury Great Pond showed an increased infection from 8% in 1999 to over 50% in 2000.

We report the discovery of an unusual flattened form of *Mer-*

cenaria mercenaria from a sand bottom in Katama Bay. It measured 57.8 mm in length, 49.3 mm in height, and 19.5 mm in width.

SUMMER FLOUNDER (PARALICHTHYS DENTATUS) PRODUCTION AT GREATBAY AQUAFARMS, INC. Nick King, GreatBay Aquafarms, Inc., 153 Gosling Road, Portsmouth, NH 03801

Since 1996, GreatBay Aquafarms (GBA) has been producing summer flounder at its hatchery in Portsmouth, NH. Recently, GBA has sent its first major crop of flounder to market from its demonstration growout facility that utilizes re-circulation technology developed by the company. Survival of fish through the hatchery phase (6 mo) tends to be less than 30% and varies with production lot. Growth of individuals differentiates following metamorphosis, and by six months considerable variation in body size occurs around the mean size of 10 g. Size grading begins at 70 days (ph) and is necessary through the growing cycle to avoid nipping and to ensure proper feed management. Currently, fish reach an average size of 650 grams in two years from hatching, and our studies have shown that growth rates differ between the sexes during commercial ongrowing. Great strides toward improving production standards and improving the growth curve for summer flounder can be obtained through advances in husbandry, nutrition, fish health, and selective breeding.

THE ECONOMICS OF NEW ENGLAND FINFISH GROWOUT: AQUACULTURE AT AN OFFSHORE SITE. Hauke L. Kite-Powell and **Porter Hoagland,** Marine Policy Center, Woods Hole Oceanographic Institution, Woods Hole, MA 02543

We have developed a bioeconomic model of an open ocean finfish growout operation. The model optimizes stocking and harvesting schedules, and projects financial flows. It allows for comparison of alternate growout sites based on their physical characteristics (distance from shore, water temperature, water depth, etc.). The model takes into account seasonal variability in the price of fish landings as well as the effect of water temperature on fish growth rates. We have illustrated the use of the model by applying it to hypothetical growout operations for cod, salmon, and flounder off the coast of New England.

The model's optimization procedure assumes that the growout operation is to produce a fixed amount of fish (v_h, by weight) each month (or in specified months only). The model determines the optimal stocking time and number of fish for each harvest month. It also calculates expected financial flows and summary values such as project NPV and the amount of up-front investment required.

For each harvest month h (h = 1,2,3,...,12), the model uses a species-specific growth function to calculate the weight at harvest of an individual fish ($f_h(m)$) stocked as a fingerling in month m (m = h-23, h-22, h-21, ...). The model then calculates the number of fish at harvest $n_h(m) = v_h/f_h(m)$, and works backward, using the mortality function, to calculate the number of fingerlings to be stocked.

For each harvest month h, the model then identifies the stocking month m that results in the maximum net revenue (discounted difference between revenue and variable cost). Revenue is the product of harvest weight and price, which varies with fish size and time of year: $v_h * p(h, f_h(m))$. (Stocking months that result in sub-market-size fish result in zero revenue and are not considered.) Variable costs include the cost of fingerlings, feed and medication, and harvesting, including associated vessel costs. The maximum net revenue determines the optimal stocking month (and length of growout), as well as the number of fingerlings.

Once stocking decisions have been optimized, the model calculates the financial performance of the growout operation month-by-month over 15 years to determine projected cash flows, project NPV, and investment capital needed, as well as operational parameters such as vessel utilization and feed volume. The table below summarizes model results for three species.

TABLE 1.

	Cod	Salmon	Flounder
number of cages	72	72	6
fingerlings/year	1,788,000	532,000	261,000
tons of feed/year	5,416	5,281	474
boat days/year	81	77	14
average harvest weight, g	1,466	4,093	1,025
harvest, tons/month	175	151	125
NPV at 5% discount rate, $m	1.50	14.16	1.07
investment capital required, $m	8.61	9.24	1.17

THE ROLE OF APOPTOSIS IN THE PATHOGENESIS OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*. Jill LaBanca, Fairfield University, Fairfield, CT 06430; **Inke Sunila,** State of Connecticut, Department of Agriculture, Bureau of Aquaculture, P.O. Box 97, Milford, CT 06460

Apoptosis, programmed cell death, is an essential part of cell renewal and embryonic development. During apoptosis, cells undergo shrinkage and zeiosis, or blebbing. Endonucleases digest DNA into 200 base pair fragments. Later, the cell breaks into apoptotic bodies that are ingested by phagocytes. Apoptosis, or the lack of apoptosis, is also pivotal in the pathogenesis of several different diseases. *Perkinsus marinus* (Dermo), *Haplosporidium nelsoni* (MSX), and Juvenile Oyster Disease (JOD) are common diseases of the eastern oyster (*Crassostrea virginica*) along the New England shoreline. The complete pathogenesis of these diseases is still unknown. We studied the effects of apoptosis on the progression of these diseases.

Archived oyster tissues from the State of Connecticut's Bureau of Aquaculture were classified according to the type of infection

and five categories were created: oysters infected with only Dermo, oysters infected with only MSX, oysters infected with only JOD, mature oysters with no infections, and juvenile oysters with no infections. Ten oysters from each group underwent *in situ* hybridization to detect DNA fragments by end labeling. A 6 μm section from each oyster was deparaffinized and digested with proteinase K. A digoxigenin nucleotide probe was used to anneal to and label the 200 base pair fragment yielded by apoptosis. The probe was detected immunohistochemically using an antidigoxigenin peroxidase conjugate. The complex was stained with a peroxidase substrate and the slide was counter-stained with methyl green.

Apoptotic hemocytes were found to be present in the gill and stomach epithelia and surrounding connective tissue at a regular rate in both sets of healthy oysters. The oysters infected with Dermo had a reduced number of apoptotic hemocytes present in their tissues. These oysters had Dermo weighted prevalences from 0.5 to 4. The prevention of hemocyte apoptosis yields a greater number of hemocytes in which Dermo houses itself. Large numbers of Dermo cells in some infected oysters were eliminated via apoptosis in the stomach epithelia, disabling the spread of infectious particles through sea water. The oysters infected with MSX also had reduced numbers of apoptotic hemocytes. MSX appears to prevent hemocyte apoptosis. In some infected oysters large numbers of MSX-plasmodia were eliminated via apoptosis. Part of the vesicular connective tissue cells of MSX-infected oysters were apoptotic. Oysters infected with JOD had the presence of large, unidentified, apoptotic cells in the stomach epithelia whose role has not yet been determined.

Apoptosis enhances progression and prevents transmission of oyster diseases. The ability of oysters to eliminate parasites via apoptosis may establish the genetic basis of disease resistance.

EFFECTS OF NURSERY CULTURE TECHNIQUE ON THE MORPHOLOGY AND BURROWING CAPABILITY OF THE SOFTSHELL CLAM, *MYA ARENARIA*. Kenneth J. LaValley, Spinney Creek Shellfish, Inc., Eliot, ME 03903

In August of 1999 trials began to evaluate the benefits of a raceway nursery system compared to a Floating Upweller System (FLUPSY). Raceways are shallow rectangular trays into which water is introduced at one end, flows over the seed clams, and exits at the other end through a drain. During normal operation, silt from the estuarine waters and pseudofeces from shellfish produce a substrate. This substrate would be detrimental to other bivalve shellfish (oysters, quahogs) and would represent an intense maintenance problem. However, to the juvenile softshell clam the substrate represents an artificial mud flat.

It was initially hypothesized that the ability to burrow into a substrate would allow enhanced growth by reducing the physiological stress experienced while in an upweller system. The anterior and posterior adductor muscles, having evolved to suit a habitat several inches beneath the sediment, are unable to maintain a "normal" gape or closed position outside of sediment. This condition results in a physiological stress, which incurs abnormal growth. During initial FLUPSY trials a significant stress (reduced growth, shell deformities) was observed after juveniles had reached 15 mm.

To determine the effect of initial size and planting density, two size ranges of softshell clam seed, 6 and 9 mm, were planted at three densities (4, 8, and 16 clams/square inch) within the raceway. In order to compare the performance between the two nursery techniques both size ranges were stocked in the floating upweller system at a density of 8 clams per square inch. The weekly growth rates were consistently higher at the raceway than the floating upweller. The raceway clams grew at a rate of approximately 2 mm per week, while the upweller clams did so at a range of .75 to 1.0 mm per week. Stocking density did not significantly affect growth rate of raceway clams.

Following growth and density comparisons, culture observations were statistically evaluated by comparing "wild" softshell clam seed to cultured animals, as well as by comparing the morphology of market size "wild" softshell clams to cultured seed. We also determined and compared the burrowing capability of "wild" and cultured seed.

Softshell clams produced in the FLUPSY significantly differed in their morphology compared to clams cultured in the raceway or "wild" seed. Clams cultured in the FLUPSY also demonstrated a significant decrease in their ability to burrow compared to raceway or "wild" seed. The percentage of clams that successfully burrowed in a two hour period was proportionally lower in seed produced in the FLUPSY (65% compared to 80%).

Raceway seed did not significantly differ from "wild" seed or market size animals in their morphology or ability to burrow. In summary, nursery technique can significantly affect the morphology and behavior of cultured shellfish. These differences may significantly impact initial survival of softshell clam seed postplanting. For softshell clams, a sediment-filled culture environment appears to be the optimal nursery strategy.

THE RAZOR CLAM (*ENSIS DIRECTUS*) AS A CANDIDATE FOR CULTURE IN THE NORTHEAST: AN INTRODUCTION. Dale Leavitt and **William Burt,** Southeastern Massachusetts Aquaculture Center, Hurley Library, Mass. Maritime Academy, 101 Academy Dr., Buzzards Bay, MA 02532

There is an urgent need for the shellfish culture industry in the northeastern United States to expand their list of candidate species for culture and to diversify their crop. Reliance on two species of bivalve mollusk, given the historic and current prevalence of debilitating diseases, may result in lost opportunities to use sites for farming shellfish. By expanding the selection of candidate species, growers will have better success in conducting their business by providing alternate crops that may be appropriate for their specific growout situation.

The razor clam represents one candidate species that has a high

potential for commercialization. Market demand for the razor clam seems to be constant and can be expanded given the relative lack of awareness on the part of consumers regarding the acceptability of the product. Landed value is currently at a level that makes farming the razor clam economically attractive. The constraints at this point are focused on developing the appropriate technology for growing the razor clam to a market size.

The razor clam exhibits biological properties that suggest it is a reasonable candidate for culture. It undergoes a routine bivalve larval cycle that should adapt to hatchery conditions readily. It grows relatively quickly and has been successfully cultured as a by-product under quahog anti-predator netting, due to wild recruitment onto the site. It naturally inhabits intertidal and subtidal areas that are currently being used for quahog culture.

The razor clam, however, will present some challenges to development of it as a commercially viable farmed bivalve. These include:

1. Mobility
2. Over-winter survival
3. Predators & disease
4. Shelf-life
5. Overall lack of knowledge about the species.

To aid in diversifying the industry, the Northeast Regional Aquaculture Center (NRAC) has funded us to begin work on developing technology for razor clam growout. The overall objective of this project is to provide an opportunity for the current shellfish culture industry to investigate, develop, and optimize the growout technology for a cultured razor clam. An overview of the projected work and a solicitation for industry involvement is presented.

PRELIMINARY STUDIES ON OPTIMAL ROTIFER CULTURE DIETS AND ALTERNATIVE REPLACEMENT DIETS AND ENRICHMENTS FOR LARVAL BLACK SEA BASS (*CENTROPRISTIS STRIATA*). **Jennie M. Mandeville,** Middlebury College, MC Box 3365, Middlebury, VT 05753; **Mark T. Watson** and **Brandy M. Moran,** Massachusetts Institute of Technology Sea Grant College Program, MIT Bldg. E38–300, 292 Main St., Cambridge, MA 02139

Rotifers *(Brachionus plicatilis)* are excellent first-feed food for cultured larval fish as they are one of the fishes' naturally occurring food sources, small in size, mobile, and easily cultured. It is important that the rotifers are fed a healthy diet that promotes rapid growth in order to maintain a constant food source for the larvae. Several rotifer diets have been tested in the past; this study examines three new, as well as previously tested, diets for their effect on rotifer population growth. The diets were Instant Algae (Reed Mariculture, CA), consisting of *Nannochloropsis oculata* (Nanno), *Isochrysis galbana* (T-Iso), and *Pavlova sp.*, bakers yeast and Algamac 2000 (Aquafauna Bio-Marine, Inc., Hawthorne, CA). Fifteen combinations of these diets were evaluated for their performance. Nanno supported the highest maximum population growth rate, and was significantly higher than 8 other diets ($P < 0.05$). However, three other combinations of algae and yeast significantly ($P<0.05$) enhanced population growth rates as well. Food combinations including Algamac resulted in significantly ($P < 0.05$) poorer growth rates. While preliminary, these findings can be used to guide aquaculturists in their choice of rotifer diet, and also as a foundation on which to base future studies exploring the nutritional value that these diets confer to larval fish.

Advancements in culture methods of alternative marine finfish species, especially early life stages, are necessary in order to encourage commercial production. Alternative diets and more efficient live feed enrichments are two ways to enhance growth and survival of cultured fish. Black sea bass *(Centropristis striata)* eggs, spawned in captivity at the University of Rhode Island (Kingston, RI), were transported to the Massachusetts Institute of Technology Sea Grant College Marine Finfish Hatchery (Charlestown, MA). The eggs were incubated in filtered (1 µ, with UV) harbor water (22 °C, 28 ppt salinity), and 24 h photoperiod) until hatch and then transferred into nine 40 L tanks.

In preliminary studies we tested a live-feed replacement, Revolution (Salt Creek Inc., UT) liquid fish diet, and compared it to rotifers enriched with Instant Algae (Nanno and T-Iso). Newly hatched larval sea bass were fed three combinations of diets: (1) 100% Revolution diet, (2) 50% Revolution and 50% Rotifers, and (3) 100% rotifers. Results of growth and survival showed that there was no difference between diets #2 & #3; diet #1, however, resulted in 100% mortality after 10 days, demonstrating that the Revolution diet can be used as a supplement in larval fish cultures, but not as a substitute for rotifers. Recent studies have shown that larvae allowed to feed for several days on live feed prior to replacement diets show higher survival. This is thought to permit proper digestive tract development.

A second series of studies compared various enrichment media for rotifers and examined their effect on growth and survival of newly hatched black sea bass larvae. Three popular, commercially available enrichments were tested: Instant Algae (Nanno and T-Iso); Culture Selco (Inve Aquaculture Inc., Grantsville, UT); and Algamac 2000. Preliminary results showed that Algamac 2000 provided the highest growth and the most consistent survival (50%) as an enrichment. Research is continuing to compare the economic benefits of the various enrichments.

PREVALENCE OF *PERKINSUS MARINUS* IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*, IN RHODE ISLAND. **Karen Mareiro, Josefa Dougal, Meggan Dwyer, Kenneth Leonard III** and **Marta Gómez-Chiarri,** University of Rhode Island, Fisheries, Animal and Veterinary Science, Kingston, RI 02881; **Arthur Ganz,** Rhode Island Department of Environmental Management, Coastal Fisheries Laboratory, Wakefield, RI 02879

Populations of Eastern oyster, *Crassostrea virginica*, along the East Coast of the United States have been severely decimated by various diseases in recent years. One of the most common of those

diseases is Dermo disease, which is caused by the protozoan parasite *Perkinsus marinus*. Since 1998, our laboratory has been tracking the prevalence of this disease in oysters from several locations in Rhode Island. Thirty oysters were collected from 12 sites throughout Rhode Island waters, including aquaculture lease sites, in August and November of 1998–2000. The presence and intensity of Dermo disease was tested using the Ray's Fluid Thioglycollate Media (RFTM) method. Dermo infections were detected at most sites, with the highest intensity of infections being observed in Barrington River and Point Judith Pond (wild). Areas where prevalence of Dermo was low include Block Island and Prudence Island. In addition, Dermo prevalence was also low in oysters collected from aquaculture lease sites. Differences in prevalence of Dermo disease in these areas could be due to lower exposure to parasite, resistance to parasite, food availability, or abiotic factors including flushing rates, salinity, and temperature variability. Furthermore, in 1998 and 1999, intensity of infections was higher in August than in November. However, in 2000 the reverse was true. It is possible that the late peak in prevalence and intensity could be due to the cold weather of early 2000 winter. This research has been funded by the Rhode Island Department of Environmental Management, Division of Fish and Wildlife.

IS *SIROLPIDIUM ZOOPHTHORUM* THE ANIMAL EATER ITS NAME SUGGESTS? NEW EVIDENCE OF PARASITISM. Christopher Martin, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

A phycomycetous fungus has been observed repeatedly in larvae of the bay scallop, *Argopecten irradians*, at the Milford Laboratory. This microorganism has been tentatively identified as *Sirolpidium zoophthorum* Vishniac, first observed at this laboratory by V.L. Loosanoff almost fifty years ago. The morphology and development of the fungus have been previously described. While apparently enzootic in scallop cultures at our laboratory, *S. zoophthorum* has not been tied directly to mass mortalities of this species. Loosanoff regarded it as parasitic in the bay scallop and in the larvae and juveniles of other bivalves. However, his evidence was largely circumstantial, i.e., conspicuous presence of "infected" larvae in cultures suffering high mortality.

Using pure cultures of *S. zoophthorum*, freshly isolated from affected scallop larvae, it has been possible to demonstrate that this fungus is one likely cause of observed mortality. Exposure of 72 h scallop larvae to suspensions of recently emerged zoospores resulted in approximately 70% mortality in 4 days. Fungal thalli were detected in up to 88% of dead larvae. Untreated controls remained unaffected. *S. zoophthorum* was successfully re-isolated from parasitized larvae, thus satisfying Koch's Postulates.

POTENTIAL EFFECTS OF CONTAMINANT EXPOSURE ON CULTURED TAUTOG, *TAUTOGA ONITIS*. Brandy M. Moran, Mark T. Watson, and **Cliff A. Goudey,** Massachusetts Institute of Technology Sea Grant College Program, MIT Bldg. E38–300, 292 Main St., Cambridge, MA 02139

MIT Sea Grant College Program has been demonstrating aquaculture in recirculating systems in the Boston Harbor since May 1998. The hatchery is located in the inner harbor within the Charlestown Navy Yard, Charlestown, MA. The Navy Yard was in full commission from 1799 to 1975. Due to the shipbuilding, equipment facility, and cordage activities conducted at the yard and the overall industrial character of the port, the surrounding seafloor sediments are contaminated by waste products such as heavy metals. The contaminated sediments and other pollution sources in the harbor may influence the water quality used in the recirculating systems. Because there was a concern about contaminant accumulation in the fish reared at the hatchery, a monitoring program began. To establish an economical and commercial hatchery in Boston Harbor, the possibility of cultured fish accumulating harmful pollutants needed to be investigated.

Tautog exclusively raised in a recirculating system from egg, spawned in July 1998, are currently 2 ½ years. Collaborating with the Massachusetts Water Resource Authority, the muscle tissues of these fish were analyzed for heavy metals and contaminants in February 1999 (7 mo), November 1999 (16 mo) and June 2000 (23 mo). The data reveal traces of heavy metals and other contaminants in fish tissue, but all levels are below FDA regulations, action levels, and recommendation levels. This demonstrates that fish being held for long periods of time in Boston Harbor water are safe for human consumption under current regulations.

Additional unique findings in the reared tautog were observed during this study. In February 2000, when the tautog were 18 mo, fertilized and unfertilized eggs were found in the recirculating system. This spawning occurrence is early according to literature on tautog reproductive strategies. Tautog are known to mature sexually between 2 and 3 y. By June 2000, some of the tautog showed signs of an enlarged abdomen and unbalanced swimming behavior. The fish were sent for a full diagnostic analysis. The tautog with swollen abdomens were all females that were not successfully reabsorbing unspawned eggs. The tautog also had gill deformities consisting of migrating chloride cells to the tip of the lamellae, fusion of lamellae, and enlarged mucus cells. The water quality of the system was stable over the 2 ½ year time span except for the ammonia, which fluctuated between 0 ppm–5 ppm.

These abnormalities are potentially associated with water quality and/or low-level contaminants. The impact of the fluctuating ammonia may have caused a stress-induced acclimation to the gills to be able to tolerate its surroundings. It is also possible that exposure to low-level contaminants for an extended period of time could cause these gill deformations. Both the stress from the ammonia and contaminants may affect the female tautog ability to reabsorb eggs that were not spent. Although the tautog are edible

under the current FDA regulations, the exposure to these contaminants could be affecting the overall health of the fish. Further research is needed to determine tautog's exposure tolerance to metals, ammonia, and other critical environmental parameters. This information would aid in the siting and success of a commercial hatchery in industrial harbors.

BOTTOMS UP! AN INDUSTRY-LED PROJECT: BRINGING AN AQUACULTURE TECHNIQUE TO THE INSHORE SCALLOP FISHERY IN MAINE. Dana L. Morse, University of Maine, Darling Marine Center, 193 Clark's Cove Rd., Walpole, ME 04573

In May of 1999, a delegation from Maine traveled to Japan to learn about the scallop culture industry. Focusing on Aomori Prefecture, the group quickly realized that the Japanese industry relied on a successful spat collection program, and that elements of this program could yield benefits in Maine's wild fishery. The late summer and fall of 1999 saw many fishermen up and down the coast experimenting with spat collection gear, particularly in the port of Stonington.

Since the start, industry has had the leading voice in implementing and continuing this work, with strong collaborations with a wide variety of agencies and organizations. Catches of seed from the 1999 year class were spotty but encouraging, and preliminary assessments from the year 2000 set indicate that catches will be good.

Many obstacles exist for the continued development and acceptance of the program. However, the effort has yielded promising results thus far, and industry support has remained strong. Future work will rely on this continued support, and from the productive working relationships with other groups in the state.

EFFECTS OF FILTER-FEEDING OYSTERS ON SEDIMENTATION RATES AND PHYTOPLANKTON SPECIES COMPOSITION: PRELIMINARY RESULTS OF MESOCOSM EXPERIMENTS. Jennifer Mugg, Michael A. Rice, and Monique Perron, University of Rhode Island, Fisheries, Animal, and Veterinary Science, Kingston, RI 02881

Eutrophication is occurring in many coastal estuaries. A possible solution to this problem is to raise aquaculture oysters to improve water clarity and to help remove excess nitrogen. In order to determine what effects aquaculture oysters have on the environment, a mesocosm study was performed at the Marine Ecosystem Research Laboratory (MERL) from June to October 2000. The MERL facility is located adjacent to Narragansett Bay with thirteen 13,000 L mesocosm tanks that simulate the environmental conditions of the Bay. Two hundred oysters (≈35 mm in valve length; nominally filtering about 48 L da^{-1} ind^{-1}) were placed into three mesocosms, and three mesocosms were maintained without oysters as controls. Experiments were run with varying rates of water exchange in the tanks ranging from 0% to 100% per day (0–13,000 L da^{-1}). Several parameters were measured and compared between the two treatments, which included chlorophyll-a, particulate organic and inorganic matter, sedimentation rates, nitrate, ammonia, phytoplankton analysis and growth rates.

Preliminary results show that oysters have an effect on species composition of phytoplankton in the water column and induce increased rates of sedimentation to the benthos. Diatoms of the genus *Nitzschia* were predominant in mesocosms with oysters, and in the control tanks *Skeletonema* were dominant. Tanks with oysters consistently showed rates of sedimentation greater than twice the control tanks. We speculate that this increased organic sedimentation by actively filter feeding oysters may contribute to increased rates of sediment deposition leading to increased denitrification in natural systems. This is work from RI-AES Project H-886, and is publication number 3857 of the College of the Environment and Life Sciences, University of Rhode Island.

LABORATORY CULTURE OF LARVAL TAUTOG: RECENT UPDATES AND CHANGES. Dean M. Perry, David A. Nelson, and **Robin S. Katersky,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

Adult field-captured tautog, *Tautoga onitis,* were spawned in the laboratory. Larvae were cultured according to standard laboratory procedures developed at the Milford laboratory using a recirculating system containing six 1140 L conical rearing tanks. During the summer of 2000, the following changes were made to our protocol which increased larval survival. These changes included increasing live feed density in the rearing system, extending the duration of rotifer feeding, and decreasing the initial larval stocking density. Larvae were fed an average of 9×10^6 rotifers per day, which is double the amount fed in previous years. They were also fed rotifers for a 25 d period compared to 14 d in prior years. With this increased larval survival we are currently monitoring growth rates of these juveniles under laboratory conditions. From December 14, 2000 to February 2, 2001, total length (mm) and biomass were measured and recorded biweekly. Specific growth rate was 0.30 mm d^{-1} and biomass increased at an average of 0.04 g d^{-1}.

DEPARTMENT OF COMMERCE AQUACULTURE—AN UPDATE OF POLICIES, PLANS AND PROGRAMS. Edwin Rhodes, Aquaculture Coordinator, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, 1315 East-West Highway, Silver Spring, MD 20910

The Department of Commerce Aquaculture Policy, signed in 1999, has created a renewed interest in aquaculture within the Department. Guidance to Commerce agencies concerning aqua-

culture has been developed that should insure that aquaculture is considered in all relevant Department programs. The chief funding increase for aquaculture came through an additional $3 million appropriation to the Office of Oceanic and Atmospheric Research that will result in a competitive distribution of $5 million in this fiscal year, and a similar amount next year. Funding from this program in the last cycle went primarily toward management and regulatory issues, but this year's priorities include a strong emphasis on research and encourages industry, government, and academic partnerships that will result in commercialization of aquaculture, and which also will contribute to the Commerce goal of a fivefold increase in U.S. aquaculture production by 2025.

Other NOAA programs that support the development of environmentally sound aquaculture include the in-house aquaculture R&D programs at our Northeast and Northwest Science Centers, and the Fisheries Finance Program that made significant loans to the aquaculture industry in 2000. NOAA has also drafted offshore aquaculture legislation that would authorize long term leases in the U.S. EEZ. Additionally, NOAA, along with other Federal partners and with stakeholder input, is developing a code of conduct for responsible aquaculture in the U.S. exclusive economic zone. NOAA is also providing some funding for offshore aquaculture development.

KEEPING GENTRIFICATION AT BAY: HOW A DEFUNCT SHELLFISH FACILITY WAS KEPT FROM THE DEVELOPERS. Gregg Rivara, Cornell Cooperative Extension of Suffolk County, Marine Environmental Learning Center, Southold, NY, 11971; **Timothy Caufield,** Peconic Land Trust, P.O. Box 1776, Southampton, NY 11969; and **Walter Smith,** P. O. Box 395, Orient, NY 11957

The loss of waterfront real estate to non-water dependent uses in the Northeast and elsewhere has made siting new shellfish mariculture operations expensive, if possible at all. The Shelter Island Oyster Company (SIOC) was formed in 1924 by Anna and John Plock Sr. By the 1960s the company had offices in Manhattan and Greenport, and a nursery/growout facility in Southold, New York. John Plock has been called a marketing genius— many types of SIOC marketing devices can today be found in online auctions. As a result of Plock's business sense, the company was well known, and plans to sell its "greenhouse" oysters by mail were featured in *National Fisherman* in the 1970's.

A number of unfortunate events led to the demise of the company in 1984. What remained of SIOC's holdings was the Southold location, a 22-acre site of buildings, artificial lagoons, tide gates, and decaying culture gear— all in all, a facility that could not be built with today's environmental regulations. After a series of failed development plans that would have precluded mariculture at the site, the Peconic Land Trust, a not-for-profit land conservation organization, and Cornell Cooperative Extension worked on a plan with the Plock family to save most of the area used for shellfish culture while limiting development to four two-acre lots.

Dedicated in 1996, the "Shellfisher Preserve" consists of 14 acres with structures and water access that are offered by the Trust as an incubator for shellfish mariculture firms. Currently there are two such tenants, both with hatchery and nursery operations in place or planned for 2001.

DEVELOPMENT OF THE NOANK AQUACULTURE COOPERATIVE IN CONNECTICUT. Karen Rivara, The Noank Aquaculture Cooperative, 100 Main St., Noank, CT 06340

The Noank Aquaculture Cooperative (NAC) is now a legal entity which will operate a commercial shellfish hatchery and nursery system. It will provide shellfish seed to our members to grow to market size. The market shellfish will then be sold through the Cooperative. In addition to operating a commercial hatchery, the Cooperative will have wet storage and a processing facility at the Noank site.

The Noank Aquaculture Cooperative (NAC) will coordinate with the Long Island Sound Regional Vocational Aquaculture School to provide hands-on learning experiences for their students. The Groton Shellfish Commission will be involved in our education outreach program. We will be working with researchers in Connecticut, both from the universities and the Bureau of Aquaculture, to develop disease resistant oysters. Future work with lobsters, finfish, and seaweeds is possible. The Groton Shellfish Commission (GSC) will be managing a portion of the shellfish seed provided by the Cooperative to the Town of Groton. This seed will be used for Recreational Shellfish Programs and for their Shellfish Restoration Programs. The GSC will most likely be helping the NAC with our public education outreach programs.

The Cooperative has received a lot of help and support from the Groton Shellfish Commission, the Noank Fire District, the Connecticut Department of Agriculture, Bureau of Aquaculture, and the USDA. With this support we should become an important asset to the Southeastern Connecticut Maritime Community and to the Connecticut Shellfish industry in general.

A COMPARISON OF THE GROWTH AND MORTALITY OF JUVENILE *ARGOPECTEN IRRADIANS* IN THE VARIOUS CULTURE METHODS EMPLOYED BY THE STUDENTS AT THE SOUND SCHOOL REGIONAL AQUACULTURE CENTER. John Roy, Amber L. Beitler, and **Kathryn R. Markey,** The Sound School, 60 South Water St., New Haven, CT 06519

In 1982, the Connecticut Department of Education contacted the University of Connecticut Sea Grant Program and the Connecticut Marine Trades Association in Essex regarding the creation

of a special curriculum for a new type of regional vocational agriculture center—one devoted to Aquaculture Science and Technology. Five sites were proposed that would cover the Connecticut coastline (no coastal towns would be excluded). The sites included Stamford for the western part of the state, then Bridgeport (Fairfield to Milford), then New Haven (West Haven to Guilford), Old Lyme (Clinton to East Lyme), and either Groton or New London for the eastern Connecticut towns. These new vocational aquaculture centers were designed not to compete with the existing agriculture centers, but to offer young people a new program based on the educated use and appreciation of the many marine business opportunities offered by the sea and coastal waters. It was supposed, at the time these schools were being conceptualized, that there would be a tremendous amount of interest from the shoreline communities. However, this has not proved to be the case. The limited inclination for communities to act as host district is perplexing.

To support the development of the program, a planning committee was created in 1983 to review site criteria and assemble a curriculum for these new "Aquaculture Centers." Vocational education was viewed as a long-term investment in young people. Economic impacts of these students would maximize 15 years after high school graduation. This had a tremendous impact on the curriculum that strived to educate both for current job titles and future aquacultural employment opportunities. The curriculum is not static. As advances in technology unfold and new scientific finds are realized, we as aquaculture teachers are challenged to upgrade and expand the curriculum.

Aquaculture as an applied science lends itself readily to the classroom. The senior science courses offered at the Sound School comply with the Vocational Agriculture Education Standard of offering students, in their fourth year at a VoAg Center, the option of continuing and concentrating their studies in the science portion of the school's curriculum. Students taking Special Topics have elected to participate in the science portion of the Aquaculture program for their entire senior year at the Sound School. As further dictated by VoAg standards, the curriculum of this class is determined by student-driven investigation. Each student who has chosen to participate in this class will select an area of interest, will create a specialized plan of study, and will pursue his or her chosen aspect of aquaculture through in-depth research.

Special Topics incorporates the Vocational Agriculture attitude of learning through doing. The students perform a variety of experiments each year. In October 2000, a study was begun with *ca* 10,000 juvenile *Argopecten irradians*. The bay scallops were purchased from Frank M. Flower and Sons Inc. of Long Island, New York. The guaranteed seed size was 25 mm. The scallops were placed in a variety of cages, open systems, and static culture. The students involved in the project have compared the effectiveness of the various methods of growout by monitoring growth and mortality.

FEDERAL CROP INSURANCE FOR QUOHOG FARMS. **John Sarkes,** Rural Community Insurance Services, P. O. Box 206, Harwichport, MA 02646

The second year of the cultivated clam pilot crop insurance program developed by the United States Department of Agriculture Risk Management Agency has begun. The pilot may run three to five years before expanding. The program is offered in 11 counties in the four states of Massachusetts, Virginia, South Carolina, and Florida. Insurance is available for *Mercenaria mercenaria* that are grown in grants located in these participating counties. Growers can check with the local Farm Service Agency office or county extension service to see if they are eligible.

A brief summary of the crop year and the overview of the program were presented. General information pertaining to insurability conditions, such as covering during the growout phase and size requirements for clams, was included. It was emphasized that mortality due to oxygen depletion, disease, freeze, hurricane, increase or decrease in salinity, tidal wave, storm surge, and windstorm are covered losses. Only losses that occurred during the insurance period are covered. It was further explained that it is the farmer's responsibility to follow good farming practices, the cultural practices generally in use in the county, and those recognized by the Cooperative State Research Education and Extension Service. A field representative explained what the loss adjuster's job is and some of the challenges encountered in the field.

AN INTRODUCTION TO THE S.P.A.T. (SPECIAL PROGRAMS IN AQUACULTURE TRAINING) INITIATIVE. **Kim W. Tetrault, Robert M. Patricio,** and **Rory MacNish,** Cornell Cooperative Extension of Suffolk County, Marine Environmental Learning Center, Southold, NY, 11971; and **Jonathan P. Polistena,** Mattituck High School, Mattituck, NY 11952

Cornell Cooperative Extension of Suffolk County, N.Y. has maintained and operated a shellfish hatchery on the North Fork of Long Island for the past decade. While the primary function of the hatchery has been to produce seed clams, scallops, and oysters for local town enhancement programs, the facility has been able to expand its goals to include a series of educational and training initiatives collectively referred to as S.P.A.T. (Special Programs in Aquaculture Training). The S.P.A.T. campaign, in its broadest interpretation, is a model that incorporates a community-based shellfish enhancement effort with intensive aquaculture training and information gathering. The principle components of the project include monthly public workshops pertaining to all aspects of shellfish aquaculture, extensive on-site test plots for nursery and growout of cultured seed products, a master shellfish gardener program, school outreach and teacher training opportunities, and a formalized internship program. An additional five-day summer program will also be available to ages 13–18, with overnight housing provided to non-residents. It is anticipated that these new pro-

grams will prove to be extremely popular and have, at this time, solicited over 100 responses.

BROODSTOCK CONDITIONING OF THE SEA SCALLOP, *PLACOPECTEN MAGELLANICUS*, WITH VARIOUS MICROALGAL DIETS. **Bethany A. Walton,** University of Maine, Marine BioResources Program, Rogers Hall, Orono, ME 04469 and Beals Island Regional Shellfish Hatchery, P.O. Box 83, Beals, ME 04611; and **Brian F. Beal,** University of Maine at Machias, 9 O'Brien Ave., Machias, ME 04654

One of the primary concerns of shellfish aquaculturists is to achieve maximum survival of larvae in culture. One possible way to achieve this goal is to obtain high quality eggs from broodstock. It has been well documented that conditioning broodstock may help to increase the number of high quality eggs released during spawning, thereby increasing larval viability. A laboratory study focusing on 40 adult sea scallops (*Placopecten magellanicus*) obtained from the Eastport, ME area was conducted between March and June of 2000. Sea scallops (mean size = 115.6 mm ± 8.85 mm SD) were placed individually into aerated buckets that held 12 L of filtered seawater. These 40 animals were examined nondestructively on a monthly basis for the condition of their gonads; gonad length and thickness were also measured. These were compared to a sample of animals obtained from the same location that were dissected to determine whether conditioning animals with microalgal diets had any effect on gonadal development. The following four microalgal treatments were used for the duration of the experiment and were fed at a rate of 55,000 c/ml per species for a total of 110,000 c/ml: (1) *Tetraselmis sp.* (MC:2) and *Tetraselmis chui*, (2) *Tetraselmis chui* and *Thallassiosira weissflogii*, (3) *Rhodomonas salina* and *Tetraselmis striata* and (4) *Tetraselmis* sp. (MC:2) and *Rhodomonas salina*. Initial temperature was maintained at 5°C ± 1°C, and slowly increased at intervals to a final temperature of 10°C ± 1°C. Preliminary results indicate that conditioning broodstock sea scallops with certain microalgal diets increases the gonad index.

REMOVAL OF BLUE MUSSELS, *MYTILUS EDULIS*, FROM MUDFLATS: DOES IT IMPROVE SOFTSHELL CLAM, *MYA ARENARIA*, HABITAT? **William C. Walton,** Beals Island Regional Shellfish Hatchery, PO Box 83, Beals, ME 04611

In response to apparent increasing abundance of the blue mussel, *Mytilus edulis*, on certain productive mudflats, municipal shellfish managers in Downeast Maine have become concerned about the effects of mussels upon clams. In Perry, ME, the shellfish committee enlisted local clam diggers to remove ~1 acre of mussels to reduce presumed negative effects upon softshell clams, *Mya arenaria*. To test both the effects of mussels and the efficacy of removal, we conducted a field-based experiment initiated on August 1st, 2000.

Sediment-filled, plastic flowerpots (~150 mm deep and ~150 mm in diameter) were each seeded with 10 juvenile clams, 14.3 ± 0.17 mm shell length. To test the effect of the presence of a mussel bed, 36 pots were then placed in either a bed of mussels (a patchwork of clumps of mussels) or on an adjacent mudflat in the lower intertidal of a cove in Perry. Within each of these two areas, pots were placed underneath a clump of mussels (>0.5 m in diameter), adjacent to a clump, or in an open area without mussels (>1 m from any mussels). Lastly, half of the pots were meshed to protect them from predation with flexible 6 mm meshing.

All pots were collected on September 28th, 2000, and surviving clams were counted and measured. Survival was significantly lower in unprotected pots ($P = 0.0001$) than protected pots, and was also low underneath mussels. More interestingly, among unprotected clams not under mussels, survival tended to be better in the mussel bed than on the flat (adjacent, $P = 0.0801$; open, $P = 0.0380$). In terms of growth, final shell length was lowest underneath clumps of mussels; among clams not underneath mussels (where survival was very low), growth tended to be better on the mudflat than in the mussel bed.

This result was greater for unprotected clams ($P \leq 0.1437$) than for protected clams ($P \geq 0.2803$), suggesting a possible interaction with predation. Thus, the presence of mussels has a mixture of effects upon clams, which need to be considered when contemplating removal.

NURTURING A SOFTSHELL CLAM PRIVATE/PUBLIC INITIATIVE ON MASSACHUSETTS' NORTH SHORE. **Scott Weston, Joseph Buttner,** and **Mark Fregeau,** Northeastern Massachusetts Aquaculture Center and Department of Biology, Salem State College, Salem, MA 01070

Commercial aquaculture is in its infancy on the North Shore of Massachusetts. Interest and support for aquaculture continue to grow as evidenced by an increasing number of restoration and enhancement initiatives that target the "Ipswich clam" (softshell clam, *Mya arenaria*). Many North Shore towns (e.g., Ipswich, Rowley, Gloucester and Essex) have sponsored public/private efforts involving shellfish wardens, advisory boards, shellfishers and the Merrimack Valley Planning Commission in innovative, volunteer projects to identify technologically effective and socially acceptable methods of seeding and managing local clam flats. The Department of Marine Fisheries and Department of Food and Agriculture (DFA) have provided permits and/or funding. Acknowledging the social and economic importance of a viable aquaculture industry on the North Shore and throughout Massachusetts, three regional centers have been created by legislative act and supported through the DFA. The Northeastern Massachusetts Aquaculture Center (NEMAC), housed at Salem State College, serves as a catalyst to forge the knowledge and experience gained through

independent efforts by North Shore communities into a regional, collaborative softshell clam initiative.

Since its official opening in April 1999, NEMAC's Cat Cove Marine Laboratory has made courses, reference materials, and outreach (extension) assistance available to aspiring/practicing aquaculturists. Perhaps the most significant contribution has been filling a void as a local supplier of clam seed. In its first year of operation, 30,000 softshell clams (3 mm) were acquired from the Beals Island Regional Shellfish Hatchery (ME) in October 1999, successfully raised by laboratory personnel and released as 10 mm spat in Ipswich in October 2000 by town officials, volunteers, and people from the MVPC and NEMAC. Currently, an estimated 500,000 softshell clams reside at NEMAC's Cat Cove Marine Laboratory. Most clams originated from the Beals Island Regional Shellfish Hatchery (300,000) or were collected from mud flats in Rowley by the shellfish warden and volunteer shellfishers (200,000). A modest number of clams (1,000–2,000) were spawned from local broodstock maintained in the Cat Cove Marine Laboratory (July 2000). As these clams attain an appropriate size they will be released onto the flats of Ipswich, Rowley, Gloucester, and Essex in the early summer and/or early fall 2001. Production and seeding of clams are expected to continue increasing as laboratory personnel gain experience and the facility becomes fully functional.

Through the input of varied stakeholders an economically feasible and environmentally sustainable aquaculture industry is gradually developing on Massachusetts's North Shore at a rate and in a manner consistent with the expectations of all collaborators, specifically through supplementing the traditional wild harvest with increasing numbers of cultured clams. Aquaculture is growing because local shellfishers and towns are supportive, technical expertise and an increasingly dependable source of seed exists, and state agencies are making significant financial and logistical commitments. The private/public approach to establish a sustainable aquaculture industry on Massachusetts' North Shore is working and could prove applicable elsewhere.

GROWTH AND SURVIVAL OF BAY SCALLOPS, *ARGOPECTEN IRRADIANS IRRADIANS*, FED *TETRASELMIS CHUI* BY TWO METHODS. James C. Widman Jr. and **David Veilleux,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

Tetraselmis chui was grown using two different methods, carboy vs. GRAMPS (Greenhouse Algal Mass Production System), and fed to bay scallops, *Argopecten irradians irradians*. Growth and survival were monitored. Algae grown by the traditional Milford carboy method utilize artificial light and artificial seawater with the addition of various nutrients, trace elements, and vitamins. GRAMPS-grown algae utilize sunlight and natural seawater from Milford CT harbor, which is UV treated and then enriched with a commercially available F/2 media. Current results indicate a slight growth advantage when using algae grown in the carboys. Increases in mean shell height ranged from 2.6–3.1 mm for scallops fed with GRAMPS-grown algae, while scallops fed carboy-grown algae increased 3.6–4.1 mm. Most surprising was the decrease in survival when scallops were fed *Tetraselmis* grown using the GRAMPS method. Survival of scallops on the GRAMPS-grown *Tetraselmis* averaged 64%, and scallops fed carboy *Tetraselmis* averaged 89%. Additional research is needed to determine the cause of the growth and survival discrepancies observed between the two growing methods.

EFFECT OF FEEDING RATION AND REGIME UPON GROWTH AND FOOD-CONVERSION OF JUVENILE QUAHOGS, *MERCENARIA MERCENARIA*, AND COMPARISON WITH BAY SCALLOPS AND EASTERN OYSTERS. Gary H. Wikfors, Jennifer H. Alix, Mark S. Dixon, and **Barry C. Smith.** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

Our previous studies have demonstrated effects of both feeding ration (how much) and regime (how often) upon growth and feed-conversion efficiency of juvenile bay scallops, *Argopecten irradians*, and eastern oysters, *Crassostrea virginica*. As this information is useful in design and scaling of land-based hatchery and nursery processes, we conducted an experiment identical to those done previously with bay scallops and oysters, this time with juvenile (3mm) clams, *Mercenaria mercenaria* (*notata* variety, generously provided by the F.M. Flower Co., Oyster Bay, NY). The feeding experiment was a factorial design in which ration, at 1, 2, 5, or 10% of clam live weight in dry weight-equivalent algal feed per day, and regime, with the daily ration divided equally into 2, 4, or 16 daily feedings, were co-varied. Our computer-controlled molluscan rearing chambers were used to accomplish the multiple daily feedings of a 50:50 mix of two high-lipid *Tetraselmis* strains, PLY429 and PLAT-P, that were cultured free of bacteria in semi-continuous carboy assemblies. The 50 clams held in each chamber were removed weekly, and growth was monitored as live weight, volume displacement, and shell size. Rations were adjusted weekly to remain consistent with live-weight increases, and the same clams were returned to the chambers. Organic weight growth (ash-free) of clams was determined after six weeks of feeding and compared with organic weight of algae fed to calculate conversion efficiency (CE) as the percentage of algal mass converted to clam tissue mass. A multifactor Analysis of Variance (ANOVA) was used to analyze main and interactive effects of the independent variables ration and regime upon the dependent variables growth and CE.

Clam survival was near 100% during the experiment. Growth of clams was significantly affected by ration, but not regime. Growth was not significantly greater than zero on 1 and 2% rations, and increased exponentially at 5 and 10% rations. CE was

affected significantly by both ration and regime, as well as by the interaction of these two variables. CE was highest at the 10% ration given as 16 daily feedings, but was only slightly over 2%, i.e., it took 50 g of algal dry weight to produce 1 g of clam flesh. These results contrast sharply both qualitatively and quantitatively with findings for scallops and oysters, in which maximal growth occurred on 5% rations, and CE values were in the range of 20–25%. Possible reasons for this contrast may include lower nutritional value of the *Tetraselmis* strains for clams than for the other bivalves, differences in initial size of individuals in experiments with different shellfish, or the fact that ash-free dry weight was used for clams, whereas dry tissue weight was used previously. Nevertheless, it appears likely that northern quahogs are far less efficient at using low levels of algal food, and require more food than other bivalves and, thus, are poorer suited to land-based, controlled nursery culture.

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ABSTRACTS OF TECHNICAL PAPERS

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*__Editor's Note:__ Since this meeting was topic-oriented rather than society-oriented, papers presented here represent those whose author(s) signified membership in the National Shellfisheries Association.

CONTENTS

NORMAL AND ALTERED GAMETOGENESIS IN THE GREEN SEA URCHIN: IMPLICATIONS FOR AQUACULTURE.

During the annual reproductive cycle, gonads of both sexes of the green sea urchin pass through a characteristic series of structural changes. These changes can be classified according to the activities of the two major populations of cells that compose the germinal epithelium. These cellular populations are either: (1) germinal cells (oogonia → fully mature ova in the ovary or spermatogonia → fully differentiated spermatozoa in the testis); or (2) somatic cells called nutritive phagocytes (NP) and present in both sexes. In an effort to help others better understand normal gametogenesis in the green sea urchin, we present an accompanying web page, which provides a detailed catalog of the appearance and functions of these two populations of cells during the following stages:

1. intergametogenesis and NP phagocytosis;
2. pregametogenesis and NP renewal;
3. gametogenesis and NP utilization; and
4. end of gametogenesis, NP exhaustion, and spawning.

This web page: http://zoology.unh.edu/faculty/walker/urchin/gametogenesis.html also covers gamete structure, fertilization, and very early sea urchin development, gives an extensive bibliography, and will be amended as new information becomes available. We also encourage others with more extensive information than our own to help us to develop this web page. Intimate knowledge of these topics in the sea urchin is useful in aquaculture, if it does no more than enhance our abilities to manipulate reproduction and development. High-quality sea urchin gonads (often called roe) are distinguished by superior size, taste, color, texture, and firmness (= extended shelf life). Ovaries and testes containing fewer gametes relative to somatic cells (NP) are preferred in most cultures that eat sea urchin gonads. Gametogenesis usually has a negative influence on the quality of sea urchin gonads. This is particularly true near the conclusion of gametogenesis, when gonads are very fragile and contain mainly gametes. Rough handling of sea urchins during harvesting results in disintegration of the gonads. In the future, a more thorough understanding of the cell and molecular biology of sea urchin gametogenesis will permit us to manipulate the process at various stages to accomplish particular marketing goals. For example, it would be advantageous for aquaculturists to understand: (1) how to suppress gametogenesis to produce high-quality gonads for consumption; and (2) how to promote gametogenesis for the increased production of seed stock. The concept of producing "designer" urchins with gametogenesis customized to particular market needs is not beyond reach. Based on our current understanding of the normal process of gametogenesis in the green sea urchin, we present methods that can accomplish both of these very different goals within a land-based facility.

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USING GIS, GPS, AND DIGITAL PHOTOGRAPHY IN SHELLFISH RESOURCE MANAGEMENT. William D. Anderson and **Guy M. Yianopoulos,** South Carolina Department of Natural Resources, Marine Resources Center, P.O. Box 12559, Charleston, SC 29422, USA. E-mail:andersonb@mrd.dnr.state.sc.us

Multiple data layers, consisting of intertidal oyster (*Crassostrea virginica*) spatial distributions, management categories, water quality, and boundary markers, all linked to attribute databases and digital photography, are used to characterize bivalve habitats and manage shellfish resources in coastal South Carolina. The three-dimensional intertidal oyster habitat, which serves as a critical nursery to resident and transient fish populations, is characterized as a keystone species in a geographic area devoid of submerged aquatic vegetation. Environmental perturbations, such as coastal development, boat wakes, nonpoint source runoff, and siltation, complicate resource conservation and management for a species chronically exploited by recreational and commercial harvesting.

This presentation describes how intertidal oyster resource assessments are conducted utilizing GIS, GPS, laser rangefinders, and digital photography. Color ArcView® maps are produced to delineate harvest areas for commercial fishermen; disseminated to recreational harvesters, and made available (in easily readable .PDF format) on the SC Department of Natural Resources' web page (www.dnr.state.sc.us/marine/regs/stateshell). Mariculture and wild stock shellfish permit interface with water bodies conditionally approved, restricted, and closed to harvest by coliform bacteria—dynamic and complex environment—its management facilitated by multiple GIS layers and linked attribute data. Digital maps depict oyster populations, water bodies closed by coliform bacteria, and shellfish management areas, all linked to commercial harvest and resource assessment data.

MOLECULAR ANALYSES OF A *PERKINSUS* SPECIES IN THE EUROPEAN FLAT OYSTER *OSTREA EDULIS*. Kathleen Apakupakul, Antonio Villalba, Sandra M. Casas, and Kimberly S. Reece, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062, USA. E-mail: kathleen@vims.edu

Members of the genus *Perkinsus* are protozoan parasites that have a subtropical distribution and infect a variety of shellfish host species. *Perkinsus* species have caused high mortality among the world's shellfish and are, therefore, the subject of intensive study to develop effective management strategies for the health of commercial shellfish fisheries. Currently, there are five recognized

species of *Perkinsus*. An infection of *Perkinsus* has been discovered in the tissues of the European flat oyster *Ostrea edulis*, a previously unreported host species.

To determine the species of parasites infecting the flat oyster, portions of the internal transcribed spacer (ITS) region and the small subunit ribosomal (SSU) RNA gene were sequenced and compared to the sequences of other *Perkinsus* species as well as to those of other protozoans. Based on these data, phylogenetic analyses showed that the parasites are a distinct sister clade to the *Perkinsus atlanticus/olseni* group. Whether or not these parasites comprise an entirely new species of *Perkinsus* or a strain of *P. atlanticus/olseni* has yet to be determined. *Perkinsus* species are known to have rather wide host selectivity. The sequences we obtained are different from any of those deposited in GenBank or published to date. If morphology data corroborate the differences seen in the molecular data, these parasites may be considered a new species of *Perkinsus*.

A *Perkinsus* genus-specific DNA probe was designed based on the alignments of the SSU rRNA sequences of all known *Perkinsus* species. In *in situ* hybridization protocols, this probe has been effective in labeling *P. marinus*, *P. atlanticus*, *P. olseni*, and *Perkinsus* species from *Chama pacificus*, *Ruditapes philappinarum*, *Macoma balthica*, and *Mya arenaria*. *In situ* hybridization was performed on histologically prepared infected *Ostrea edulis* tissue sections. We found that this probe also hybridizes to the parasites infecting *O. edulis*.

VARIATION IN SERINE PROTEASE GENE(S) AMONG *PERKINSUS MARINUS* ISOLATES. Gwynne D. Brown and **Kimberly S. Reece,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062, USA. E-mail: gbrown@vims.edu

Perkinsus marinus, the causative agent of dermo, is a major pathogen of the eastern oyster, *Crassostrea virginica* along the U.S. Atlantic and Gulf coasts. Although scientists have studied the organism for fifty years, little is known regarding the pathogenic mechanisms of the protozoan. Serine proteases present in cell-free supernatants of *P. marinus* cultures *in vitro* are currently under investigation as a putative virulence factor. Serine proteases have been found to play key roles in pathogenesis of several parasitic protozoans. In this study, we identified and sequenced a serine protease gene from *P. marinus*. We also examined variations in the identified gene among different isolates.

We first amplified a 475 bp subtilisin-like serine protease gene fragment from *P. marinus* using "universal" degenerate primers. The 475 bp fragment was then labeled with digoxigenin and used to screen a *P. marinus* λ phage genomic library. DNA from hybridizing phage was isolated and subjected to Southern blot analysis. Two different recombinant clones were identified. Clones were subcloned and sequenced using internal primers. Within one subclone, a 1,254 bp open reading frame was identified containing the probe sequence. BLAST analysis confirmed the similarity to subtilisin proteases, reporting an E value of 7e-40.

Genomic Southern blot analysis using the 475 bp digoxigenin-labeled probe showed that variations exist among different isolates of *P. marinus*. Two clonal isolates from Louisiana, LA 5-2 and LA 10-1, produced similar hybridization patterns with *Nci* I and *Sal* I; whereas, additional bands were detected in the isolates P-1 (York River, VA) and MA 2-11 (MA). These results suggest that either the P-1 and MA 2-11 genomes contain a second gene or that they possess an alternate allele of the same gene.

GENE TRANSFER IN CELL CULTURE OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*. John T. Buchanan, Ta-Chi Cheng, S. Reyna Gonsalves, Jerome F. La Peyre, Richard K. Cooper, and **Terrence R. Tiersch,** Department of Veterinary Science, Louisiana State University Agricultural Center, Louisiana Agricultural Experiment Station, Baton Rouge, LA 70803, USA. E-mail: jbuchanan@agctr.lsu.edu

The eastern oyster *Crassostrea virginica* supports a valuable industry along the East and Gulf coasts of the United States. Recently the oyster industry has been injured by disease problems, especially infections of the protozoa *Perkinsus marinus* (causing dermo) and *Haplosporidium nelsoni* (causing MSX). Both protozoa have been significant factors in the decline of the oyster fishery in the Chesapeake Bay. Along with techniques such as selective breeding and ploidy manipulation, gene transfer may be an effective tool to combat these disease problems.

Cell culture serves as an effective model for *in vivo* work, and gene transfer in cell culture would be useful for screening promoters and genes for use *in vivo*. Because a cell line from bivalve mollusks has not been established, it is necessary to work with primary cultures of oyster cells. To this end, ventricle cells from *C. virginica* were isolated, and conditions for optimal gene transfer and gene expression were investigated. Cells were cultured in 96-well plates in 100 μL of medium LA-2 at 4×10^5 cells per well.

The transfection reagent Effectene® (Qiagen Inc., Valencia, CA) was used to deliver DNA to cells, and expression of the reporter gene luciferase was used to evaluate promoters suitable for use in oyster cells. One of the promoters tested, the heat shock promoter (HSP70) from the snail *Biomphalaria glabrata* was found to be inducible in oyster cells, with significantly higher levels of luciferase detected after transfection and heat shock ($P < 0.00001$). The optimal conditions for heat shock were 40°C for 1 h ($P < 0.05$). Gene expression varied over time, with highest levels of expression observed 16 to 24 h after heat shock ($P = 0.0001$). Various conditions for transfection with Effectene® were tested, and optimal conditions per well were 0.3 μg DNA mixed with 3 μL of Effectene® ($P < 0.05$). Although gene expression was de-

tected, we found significant toxicity associated with transfection with Effectene® ($P < 0.05$). Other transfection reagents may provide adequate levels of gene expression in oyster cell culture with less toxicity. We have also observed luciferase expression using the following promoters: *C. gigas* actin, *Drosophila melanogaster* actin, *D. melanogaster* heat shock, and human cytomegalovirus (CMV) immediate early promoter. Future work will involve characterization of gene expression with these promoters and identification of promoters suitable for constitutive and inducible gene expression *in vivo*.

THE INFLUENCE OF SUBSTRATES AND CULTURE MEDIA FORMULATIONS ON THE ATTACHMENT AND SPREADING OF EASTERN OYSTER CELLS IN PRIMARY CULTURES. John T. Buchanan, Y. Li, and **Jerome F. La Peyre,** Department of Oceanography and Coastal Sciences, Louisiana State University, Baton Rouge, Louisiana 70803, USA. E-mail: jbuchanan@agctr.lsu.edu

The utility of cell culture in research has long been recognized. However, cell lines from any bivalve mollusk have yet to be established. In addition, a lack of standard protocols for primary cell culture has hindered bivalve research. Improved media have been recently developed in our laboratory. Enhanced cell attachment and spreading in primary culture would improve protocols and may prove essential for the development of a bivalve cell line. To this end, ventricle cells from *Crassostrea virginica* were isolated and the attachment factors collagen I, collagen IV, fibronectin, laminin, and poly-D-lysine were tested for their ability to promote cell attachment and spreading in the media JL-ODRP-4, LA-2, and LA-5. Also tested were two commercially available uncoated tissue culture plates (Falcon, Inc. and Costar, Inc.). A spectrophotometric assay (MTS assay) based on the reduction of tetrazolium salts to a formazan dye by metabolically active cells was employed to estimate metabolic activity and cell numbers remaining in a well after washing. A fluorescent assay (CyQuant assay) measuring the amount of nucleic acid in a well was used to estimate cell number in wells after washing, cell survival, and RNA production on the various substrates. Cell response to each attachment factor was measured at 1.5 d, 3.5 d, and 6.5 d.

Measured with the MTS assay, cell attachment was significantly affected by length of culture, media, and substrate ($P < 0.0001$). Interaction between media and substrate was significant ($P < 0.0001$). At day 1.5, significantly greater attachment was detected in JL-ODRP-4 on poly-D-lysine ($P < 0.05$), in LA-2 on fibronectin, poly-D-lysine, and uncoated Falcon substrates ($P < 0.05$), and in LA-5 on fibronectin, poly-D-lysine, laminin, and uncoated Costar substrates. At day 3.5, significantly greater attachment was detected in JL-ODRP-4 on all substrates other than laminin ($P < 0.05$), in LA-2 on all substrates other than laminin ($P < 0.05$), and in LA-5 on all substrates other than collagen I. At day 6.5, significantly greater attachment was detected in JL-ODRP-4 on the uncoated Costar substrate, in LA-2 on poly-D-lysine, uncoated Falcon, and uncoated Costar substrate, and in LA-5 on the uncoated Costar substrate.

Measured with the CyQuant assay, cell attachment was significantly affected by length of culture, media, and substrate ($P < 0.0001$). Interaction between media and substrate was significant ($P < 0.0001$). Results were similar to results for attachment with the MTS assay. Cell survival and RNA production were significantly affected by length of culture, media, and substrate ($P < 0.0001$), with significant interaction between these three factors.

In summary, cell attachment, cell survival, and RNA production were all significantly affected by the media and the substrate cells were cultured with. Furthermore, the response of cells to the various substrates tested varied with the media used indicating potential interaction between media components and substrates.

OPTIMIZATION OF GENE DELIVERY FOR IMPROVED OYSTER HEALTH. John T. Buchanan, Jerome F. La Peyre, Terrence R. Tiersch, and **Richard K. Cooper,** Department of Veterinary Science, Louisiana State University Agricultural Center, Louisiana Agricultural Experiment Station, Baton Rouge, LA 70803. E-mail: jbuchanan@agctr.lsu.edu

In Louisiana, over 300,000 acres of bottom area are privately leased for production of the eastern oyster *Crassostrea virginica*. Recently, disease problems have plagued this industry, particularly the protozoan parasite *Perkinsus marinus* (causes dermo disease). The transfer of human pathogens (such as *Vibrio vulnificus*) from oysters to human consumers has become a serious concern for the industry as well. Research in disease resistance and microbial elimination in oysters is needed. Along with selective breeding and ploidy manipulation, gene transfer research may lead to advances in this area.

There are unique needs associated with culture of oysters in the laboratory, especially for transgenic research. Consideration must be given to experimental replication, avoidance of contamination, and containment of genetically modified organisms. With this in mind, all of our work is done with artificial seawater in recirculating systems over seventy miles from the nearest coastal area. We have developed techniques for reliable production of high-quality oyster gametes and larvae in these conditions.

We have successfully transferred genes to oyster gametes and embryos and have observed expression of the red-shifted green fluorescent protein gene (*rsGFP*) in the oyster larvae produced. We have also observed expression of *rsGFP* in the hemocytes of transfected adult oysters, although the levels of gene expression were low (~1% of cells). We are currently screening promoter elements to identify constitutive and inducible promoters useful for expression of disease resistance genes. Because cell culture is a useful model for *in vivo* research, we have developed conditions

for gene delivery and gene expression in primary cultures of oyster ventricle cells. Using this model, we are screening the following promoters for expression in oyster cells: *C. gigas* actin, *Drosophila melanogaster* actin, *D. melanogaster* heat shock, *Biomphalaria glabrata* heat shock, *Autographa californica* nuclear polyhedrosis virus immediate early, human cytomegalovirus (CMV) immediate early, and simian virus 40 immediate early promoter. With identification of suitable promoters, we will further optimize conditions for gene transfer to oysters and begin to screen disease resistance genes in oyster cells. Combined with ongoing research in cryopreservation of oyster gametes and larvae and production of sterile oysters, progress is continuing toward the production of transgenic eastern oysters with enhanced disease resistance.

TOWARD CREATION OF A CELL LINE IN *CRASSOSTREA VIRGINICA*. **Jane C. Burns, James D. Moore, Chisato Shimizu, Hiroko Shike,** and **Carolyn S. Friedman,** University of California San Diego, 9500 Gilman Dr.-0830, La Jolla, CA 92093-0830. E-mail: jcburns@ucsd.edu

Immortalized cell lines are important tools for the *in vitro* study of the cellular and molecular biology of an organism, for the assessment of environmental toxins, and for the growth and study of intracellular pathogens. Many vertebrate cell lines were created by the introduction of oncogenes whose products interact with cell cycle regulatory proteins. To assess the feasibility of this approach in oyster cells, we tested the ability of retroviral vectors with a substituted envelope glycoprotein to infect and express foreign genes in primary cultured cells from hearts of *C. virginica*. To provide nutritional support for the cultured cells, we analyzed components of *C. virginica* hemolymph and assessed DNA synthesis of cells cultured in different media and at different temperatures.

Previous promoter comparisons in *C. gigas* had demonstrated that the Moloney murine leukemia virus (MoMLV) long terminal repeat (LTR) mediated the highest levels of reporter gene expression in cultured oyster cells. Therefore, the vector LLRNL (LTR-luciferase-RSV LTR-neomycin phosphotransferase-LTR) was used for all experiments in *C. virginica*. Vectors lacking an envelope glycoprotein ("bald vectors") are noninfectious and were used as a negative control. Oncovectors were created that expressed either the SV40 large T antigen or h-ras from the MoMLV LTR. Hemolymph was analyzed for free amino acids, organic acids, carbohydrates, metals, electrolytes, pH and osmolality. DNA synthesis was assessed by ^{3}H-thymidine uptake.

Luciferase expression was detected in the cell lysates of 100% of cultures exposed to infectious LLRNL, but in none of the cultures exposed to bald LLRNL. Luciferase activity was proportional to the number of vector particles, was highest during the first week after initiation of cultures and declined thereafter. The polycation, polybrene, had no effect on infection efficiency. Attempts to use the green and red fluorescent proteins were hampered by a high background autofluorescence in dead or dying cells in the cultures.

Vectors expressing the SV40 large T antigen and h-ras from the MoMLV LTR were tested in mammalian cell lines. Immunofluorescent staining with monoclonal antibodies to these proteins confirmed expression in transduced cells. These vectors were used to infect primary cultured heart cells. No immortalized phenotype was seen after infection of approximately one hundred twenty-six cultures from eighty-nine oysters. Attempts to infect oyster embryos are in progress. Future experiments will be directed at verifying stable integration of the vector in the oyster genome, testing whether SV40 large T binds to homologs of p53 and Rb in oyster cells and exploring the use of other cell cycle regulatory proteins (e.g. cyclins) to force cultured cells back into the cell cycle. We will also continue ^{3}H-thymidine studies to optimize nutritional support for potentially transformed cells.

THE ECOLOGICAL STRUCTURE AND FUNCTION OF INTERTIDAL CREEK ECOSYSTEMS: HOW MUCH DO OYSTERS CONTROL? **David Bushek, Richard Dame, Dennis Allen, Alan Lewitus, Eric Koepfler,** and **Don Edwards,** Baruch Marine Field Laboratory, Baruch Institute for Marine Biology and Coastal Research, University of South Carolina, P.O. Box 1630, Georgetown, SC 29442, USA. E-mail:bushek@sc.edu

Along the southeast Atlantic coast of North America, oysters form extensive intertidal reefs that provide habitat for a variety of estuarine organisms (both resident and transient), passively and actively filter particulates from the water column and facilitate nutrient cycling. Recognizing these activities, restoration of oyster populations is being promoted to improve ecosystem health in addition to revitalizing oyster fisheries. Ecosystems include a complexity of organisms that interact through a multitude of processes in space and time. To examine the role of oysters in the structure and function of intertidal creek systems, we characterized eight small (100–400 m long) intertidal creeks within the pristine North Inlet Estuary, SC, then experimentally removed oyster reefs from four of the creeks and monitored changes through time.

Despite clearly important roles of oyster reefs, their removal did not significantly alter nutrient concentrations, over-all nekton utilization, or phytoplankton production. Slight increases in oyster growth and recruitment were observed and interpreted as indications that oyster populations were near carrying capacity. Remineralization of ammonium by oysters was insufficient to satisfy primary production, but remineralization by nekton, the microbial loop and sediments more than accounts for the deficit. This observation was interpreted as an indication of redundancy in the system. Results also indicated that the biomass of nekton utilizing the intertidal creeks during summer often exceeded oyster biomass, at times by 50-fold. A general shift in phytoplankton communities from flagellates in summer, a preferred food by oysters,

to diatoms in winter corresponded to the seasonal arrival and departure of nekton.

Healthy oyster reefs undoubtedly serve important roles in estuarine ecology. At the scale of intertidal creeks within a larger estuarine ecosystem; however, the effect of removing oyster reefs on nutrient concentration is compensated by other components within the system. Impacts may be quite different at larger scales. We argue that dense bivalve reefs and beds are indicative of intense positive feedback loops that make an ecosystem resilient to small-scale change, but fragile and susceptible to large-scale change. The response is not linear across scales. Such changes have not been reported for natural systems, but are found in systems influenced by over-fishing, nutrient loading, and pollution. Thus, the management of sustainable fisheries in coastal ecosystems requires an understanding of the ecosystem science and the realization that estuarine ecosystems exhibit complex responses that are not easily explained by linear dynamics.

DISEASE RESISTANCE AND POTENTIAL BIOCHEMICAL CORRELATES IN A SELECTIVELY BRED OYSTER STRAIN. Gustavo W. Calvo, Christopher G. Earnhart, and **Stephen L. Kaattari,** Department of Environmental Science, School of Marine Science, College of William and Mary, Gloucester Point, VA 23062, USA. E-mail: calvo@vims.edu

In recent years, DEBY oysters, a VIMS stock that has been selectively bred for five generations at a disease endemic site in the lower York River, Virginia, has consistently shown higher growth, survival, and disease resistance relative to other stocks. For example, F4 DEBY outperformed offspring from two putatively disease-resistant wild Chesapeake Bay brood stocks tested over a range of salinity at three sites during 1997–1999. To understand the mechanism of disease resistance in DEBY better, we recently started a comparative study in which susceptible (Rappahanock River = RR, Mobjack Bay = MB) and resistant (F5 DEBY and CROSSBreed = XB) oysters are being exposed to *P. marinus* and *H. nelsoni* at the York River site. Brood stocks were spawned in July 1999, and juvenile oysters (n = 1,000 of each group) were deployed in replicate floating mesh cages in October 1999. In May 2000, we started bimonthly sampling to assess survival, growth, disease status, plasma protein composition and concentration, plasma proteolytic activity, and plasma inhibitory activity against *P. marinus* extracellular proteases (ECP). We hypothesize that plasma factors enumerated above are related to disease resistance. Disease and plasma factors are being examined in samples (n = 20) of each group. *P. marinus* and *H. nelsoni* are being diagnosed, respectively, by the body burden assay and by paraffin histology. Protein content is being determined by the BCA assay. Proteolytic activity and inhibitory activity against *P. marinus* ECP are determined using Hyde Powder Azure methods.

By July 2000, mean cumulative mortality was high (52%) in susceptible RR and 16% in the other susceptible group (MB). In comparison, cumulative mortality was low (3%) in resistant groups (XB and DB). Growth rate was low in RR relative to that of the other three groups. No *P. marinus* and presence of *H. nelsoni* were similarly observed in all groups in May. By July 2000, *H. nelsoni* infections were very high (96% prevalence with mostly high intensity infections) in RR. At that time, initial *P. marinus* infections of light intensity (<10 cells/g) were observed in all groups. Mean protein concentration decreased in all groups, from 13.4–16.3 mg/mL in May to 2.1–9.1 mg/mL in July, with RR exhibiting the highest decline. Mean proteolytic activity decreased, particularly in RR, during the same time interval. Inhibitory activity against *P. marinus* ECP was extremely variable (SD > mean) in all groups. These preliminary observations suggest that *H. nelsoni* has an adverse effect on protein content and proteolytic activity in susceptible oyster stocks.

A MODEL OF WITHIN-HOST POPULATION DYNAMICS OF THE OYSTER PARASITE *PERKINSUS MARINUS*: SIMULATED EFFECTS OF TEMPERATURE AND SALINITY. Lisa M. Ragone Calvo and **Eugene M. Burreson,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, Virginia 23062. E-mail: ragone@vims.edu

A computer model was developed to investigate the population dynamics of the protistan parasite, *Perkinsus marinus*, within its host, the eastern oyster, *Crassostrea virginica*. The individual-based model, which is driven by temperature and salinity, tracks average within-host parasite density in oysters, age one year and older. The model was validated using parasite abundance and environmental time series from three oyster populations located along a salinity gradient in the James River, VA. Predicted parasite abundance exhibited a dynamic steady state and significantly correlated with actual observed densities at all three locations (Fig. 1). The simulations accurately captured the distinct seasonal periodicity of *P. marinus* and the relative differences in abundance that typically occur along a salinity gradient.

We are presently using the model to examine the interaction of temperature and salinity in controlling the initiation and termination of *P. marinus* epizootics. In preliminary experimental simulations, parasite extinction occurred in up-river oyster populations following a single year of "normal," long-term average, salinity, and temperature conditions, when no new infections were acquired. In contrast the parasite persisted at a down-river location for the entire ten-year simulation period, despite the fact that no new infections were acquired and that temperature and salinity were consistently "normal." This suggests that a single transmission event may be sufficient for *P. marinus* to become enzootic in specific year classes of oysters located in moderate-to-high salinity areas, whereas, periodic transmission events are required for the parasite to persist in lower salinity areas.

Simulation results indicate that fairly accurate quantitative predictions of *P. marinus* abundance can be made using *in situ* tem-

perature and salinity data and a relatively simple model. An improved understanding of *P. marinus*–environment interactions will aid oyster management and rehabilitation efforts.

DETECTION OF THE FLAT OYSTER (*OSTREA EDULIS*) PARASITE *BONAMIA OSTREAE* BY FLUORESCENT *IN SITU* HYBRIDIZATION. **Ryan B. Carnegie,[1] Bruce J. Barber,[1]** and **Daniel L. Distel[1,2],** [1]School of Marine Sciences and [2]Department of Biochemistry, Microbiology, and Molecular Biology, University of Maine, Orono, ME 04469, USA. E-mail: ryan.carnegie@umit.maine.edu

Reliable and sensitive detection of parasites is essential for prevention and management of disease in shellfish aquaculture. Of the two common molecular diagnostic techniques complementing standard histopathology, *in situ* hybridization (ISH) assays hold the advantages over polymerase chain reaction (PCR) assays of providing a morphological validation and a physical context; that is, a tissue location, for a positive signal. The development of an ISH assay using fluorescently labeled DNA probes for the flat oyster parasite *Bonamia ostreae* was the objective of this project.

Three fluorescein-labeled, *Bonamia ostreae*-specific oligonucleotides, when hybridized in repeated single reactions to paraffin-embedded *Ostrea edulis* sections, reacted specifically with *B. ostreae* ribosomal RNA. Nonspecific hybridization to *O. edulis* cells or tissues was never observed.

A fluorescent *in situ* assay for *Bonamia ostreae* has several advantages over a nonfluorescent analog. An epifluorescence microscope is necessary to view the slides, but otherwise FISH is faster and less expensive than ISH with digoxigenin-labeled or biotinylated probes, which require additional treatments for visualization. Most significant will be the ability to employ multiple stains. Fluorescent probes specific to different symbionts or parasites, or different strains or subspecies of the same parasite, may be distinguished on a single tissue section when hybridized simultaneously. Such fluorescent assays will, thus, be powerful tools for the diagnosis of shellfish parasites and for studies of the epizootiology of these organisms.

SUMMER MORTALITY OF THE PACIFIC OYSTER *CRASSOSTREA GIGAS*: INFLUENCES OF CULTURE METHODS, SITE CONDITIONS, AND STOCK SELECTION. **Daniel Cheney, Ralph Elston, Brian MacDonald, Kendra Kinnan,** and **Andy Suhrbier,** Pacific Shellfish Institute, 120 State Ave NE #142, Olympia, WA 98501, USA. E-mail: psi@pacshell.org

During the late summer to early fall period, Pacific oysters cultured on the West Coast of the United States and elsewhere may experience high levels of mortality. In the 1960s to the 1980s, this condition was subject to intensive investigation focusing on broad areas of disease pathology, genetics, physiology, and the environment. Results of these studies were largely inconclusive, or pointed to a poorly defined etiology. Although several factors, such as a bacterial and herpes-like virus infections could be linked to certain mortality events, no clear picture emerged.

Recent studies in Puget Sound, Washington, USA and Tomales Bay, California, USA center on the influence of multiple stressors and their effects on oyster survival, physiology, and pathology. The goal of this research is to identify possible modifications in culture practices, brood stock selection, or grow-out location to increase survival of Pacific oysters.

Field observations indicate oysters are subject to extreme variations in a number of parameters during intertidal cycles. Annual or seasonal variations in those parameters and differing culture practices likely play major roles in oyster survival. An increased rate of oyster mortality and modified physiological response seems to be strongly correlated with both elevated temperatures and extended periods of depressed dissolved oxygen (DO). A long period of neap tides with low and slack water during the evening was observed to result in daily and successive reductions in DO to levels, ranging from 0.5 and 2 mg/L. The DO reductions are sometimes coupled with heavy macroalgae blooms and high phytoplankton densities. This and other work also indicate oyster summer mortality rates are strongly influenced by ploidy and broodstock origin/stock selection. These observations have renewed interest in testing stocks selected for reduced rates of summer mortality and that retain desirable characteristics of good growth and meat yield.

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***DE NOVO* FATTY ACID SYNTHESIS IN *IN VITRO PERKINSUS MARINUS* MERONTS: AN IMPLICATION OF BITROPHIC METABOLIC PATHWAYS IN MERONT STAGE.** **Fu-Lin E. Chu, Philippe Soudant,** and **Eric Lund,** Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, P.O. Box 1346, 1208 Greate Road, Gloucester Point, VA 23062, USA. E-mail: chu@vims.edu

Perkinsus marinus is a protozoan parasite of the Eastern oyster, *Crassostrea virginica*. Meront stage *P. marinus* isolated from infected oysters have a fatty acid composition similar to the host. They are both rich in n-3 polyunsaturated fatty acids (PUFAs) as is typical of marine organisms. However, when meronts are cultured in a host-free media containing n-3 PUFAs they contain low levels of n-3 fatty acids and high levels of n-6 fatty acids which are characteristic of terrestrial origins. These results suggest that *P. marinus* meronts may have bitrophic metabolic pathways: utilizing host lipids when host-associated, but synthesizing their own lipids when they are not host-associated. To test whether *P. marinus* meronts are capable of synthesizing fatty acids *de novo*, we incubated meronts for 48 h in seawater containing acetate labeled with

deuterium and ^{13}C, or deuterium-labeled palmitic acid (16:0). GC/MS analysis of fatty acid methyl esters (FAMEs) from meronts incubated for 48 h with acetate revealed isotope incorporation in palmitic acid, 16:0. Molecules of 16:0 contained up to 62% labeled C and H originating in acetate. Analysis of samples incubated with deuterium labeled 16:0 found isotope incorporated in 18:0, and 18:1(*n*-9). This provides evidence that *P. marinus* meronts are capable of *de novo* synthesis, elongation, and desaturation of fatty acids. The results suggest that host-free *P. marinus* meronts are capable of synthesizing a range of fatty acids from dissolved organic matter. This capacity may play a role in the survival of *P. marinus* outside the host and the parasite's ability to use the water column as a transmission vector for colonizing new oyster populations.

TABLE 1.

Evidence of fatty acid synthesis, elongation, and desaturation by *Perkinsus marinus* from stable isotope metabolism.

Substrate	Product	MW-Labeled FAME*	MW-non-labeled FAME
Sodium acetate 1,2-$^{13}C_2$;D_3	16:0	300	270
Palmitic acid D_{31}	18:0	329	298
Palmitic acid D_{31}	18:1	325	296

* Molecular weights (MW) of fatty acid methyl esters (FAME) from *P. marinus* cells incubated in York River water (28 ppt) with stable isotope substrates for 48 h. Products were identified and their masses determined by GC/MS.

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PRODUCTION AND BINDING SPECIFICITIES OF MONOCLONAL ANTIBODIES TO *PERKINSUS MARINUS* CELLULAR ANTIGENS. Christopher F. Dungan and **Rosalee M. Hamilton,** Cooperative Oxford Laboratory, Maryland Department of Natural Resources, 904 S. Morris Street, Oxford, MD 21654, USA. E-mail: cdungan@dnr.state.md.us

Availability of polyclonal antibodies labeling *Perkinsus marinus* cells and secretory products fostered development of several novel and rapid immunoassays for sensitive detection of this lethal oyster pathogen in oyster tissues, tissue extracts, and environmental waters. Although differentiation of species within the protozoan genus *Perkinsus* remains equivocal, rabbit polyclonal antibodies to *P. marinus* werealso found to label all described *Perkinsus* species as well as several tested dinoflagellates. Production of monoclonal antibodies (MAB) with narrowed specificity for *P. marinus* was conducted to produce unlimited quantities of diagnostic antibodies for monospecific detection and identification of this important oyster pathogen.

Soluble cytoplasmic, detergent-extracted, and whole cell immunogens from *in vitro P. marinus* cells were used to hyperimmunize donor mice to produce B-lymphocytes employed in cell fusions for production and cloning of immortal hybridoma cell lines secreting MABs to *P. marinus* cellular epitopes. Resulting hybridomas were screened by enzyme-linked immunosorbent assays (ELISAs) for secretion of specific IgG isotype immunoglobulins, using solubilized parasite proteins immobilized on polystyrene microtiter plates, and positive cell populations were cloned at least three times by limiting dilution plating. Of twelve different hybridomas cloned and cryopreserved, ten secrete monoclonal IgG_1 antibodies, and two secrete IgM.

IgG_1 MABs were produced *in vitro* for isolation from tissue culture medium by ultrafiltration and thiophilic gel chromatography. Binding specificities of purified MABs determined by Western blot analyses of reduced and native proteins indicate that a variety of different molecular weight epitopes are recognized by several MABs, but that the epitopes recognized by most are labile under reducing PAGE conditions. Similarly, MAB immunostaining of fixed parasite cells in histological sections was variable, suggesting that some MAB binding epitopes may be corrupted by formalin fixation or histological processing. Diagnostic specificity and sensitivity testing of IgG_1 MABs to *P. marinus* are ongoing.

PHYSIOLOGICAL CONDITION AND ENERGY RESERVES IN "NATURAL DERMO RESISTANT" OYSTER STOCKS. Vincent G. Encomio, Shawn Stickler, and **Fu-Lin Chu,**Virginia Institute of Marine Science, School of Marine Sciences, College of William and Mary, Gloucester Point, VA 23062, USA. E-mail: vge@vims.edu

The objective of our study is to use physiological and biochemical indices of condition to compare performance in putative "dermo resistant and nonresistant" oysters (*Crassostrea virginica*). We compare the effects of parasitic stress (*Perkinsus marinus*) on physiological condition and energy reserves between F1 progeny from presumably genetically distinct oyster populations. These oysters represent geographically disparate populations (three Gulf of Mexico and three Chesapeake Bay populations and one hatchery strain) exhibiting variation in tolerance to the protozoan parasite *P. marinus*. These oysters were deployed in the fall of 1999 at two sites within the Chesapeake Bay, (Port Kinsale,Wicomico River; Regent Point, Rappahannock River) in areas where dermo disease is known to occur, but not MSX. Energy reserves (glycogen, lipids, protein contents) are quantified and evaluated for their contribution to over-all physiological condition. Initial analysis on all the deployed stocks showed that the Rappahannock River stock have the fastest growth. Tissue dry weights of this stock increased significantly over time at both locations, but most significantly at Regent Point. Glycogen and protein content [mg/g dry weight (dw)] increased significantly over periodic samplings, but site-specific differences were not significant. Total lipid content (mg/g dw) also increased with growth, but not significantly. However, lipid class analysis showed a significant decrease in triacylglycerol (TAG) content from May/June to July, possibly indicating a spawning event occurring before sampling. Our study provides

information on the variation in growth and condition between sites and tests interactions of environmental factors with disease progression and intensity. Biochemical data from individual oysters are correlated with changes in shell height, condition index, and *P. marinus* infection. In addition, interaction of seasonal variation in physiological condition (because of changes in temperature, salinity, and gametogenic processes) with onset and increasing prevalence and intensity of disease are examined. Biochemical analysis on other oyster stocks is currently in progress. This research was funded by the NOAA-Virginia Sea Grant–Oyster Disease Research Program.

CAN BIVALVES BE USEFUL INDICATORS OF ECOSYSTEM CONDITION? **William S. Fisher,** U.S. Environmental Protection Agency, ORD/NHEERL, Gulf Ecology Division, 1 Sabine Island Drive, Gulf Breeze FL 32561, USA. E-mail: fisher.william@epa.gov

Numerous management decisions are made to sustain multiple, and often competing, products and services from coastal ecosystems. Scientific support for these decisions emanate from environmental indicators or selected measurements used in a monitoring program. Indicators are surrogates, estimates, or representations of conditions that are otherwise too numerous or complex to measure. One of the major challenges for indicator development is to identify those measurable environmental characteristics that are essential to the integrity of valued resources.

Integrity and sustainability of ecosystem condition are important societal values that can be broadly interpreted because of the multiple products and services derived. Even scientific descriptions of ecosystem condition vary, but generally include *vigor* (function, activity, metabolism, primary production), *organization* (biodiversity, trophic structure, degree of specialization), and *resilience* (ability to resist or recuperate from stress). Indicators of ecosystem condition must reflect these qualities. Measurements on bivalves are reasonable indicator candidates, because bivalves contribute significantly in these critical ecological areas. Water filtration for feeding is requisite for metabolic activity and also generates nutrient cycling that stimulates primary production (vigor). Accumulation (and possible deactivation) of toxins, contaminants, and pathogens without adverse effects is a byproduct of water filtration and may contribute to ecosystem resilience. Bivalve shell and reef structure provide habitat for both micro- and macrofauna and flora, drawing foragers and predators into a reef-based community (organization). Also, organization is served when bivalve reef structures alter hydrology to create and/or protect habitat diversity.

It follows that the critical ecological aspects of bivalves are water filtration capacity and reef structure, characteristics that should be reflected if measurements on bivalves are to be useful indicators of ecosystem condition. A possible strategy would be to estimate gross water filtration by monitoring every (5–10 years) the number of living bivalves on a target reef, their size, and filtration rate. Reef structure might be characterized using size, shape, and biodiversity measures. Annual measurements, such as mortality rates, disease prevalence, defense activities, recruitment, or shell growth could be used as "early warning" indicators of change in the health of the population. However, physiological measurements of bivalves include a high degree of variability because of season, reproductive status, salinity, temperature, and geographic (genetic) stock. This variability can be reduced by monitoring of individual reefs (rather than across regions) and by monitoring during winter when organisms exhibit a more stable physiological condition and have lower disease intensity.

SPECIES DESIGNATION AMONG SYMPATRIC OYSTERS *CRASSOSTREA ARIAKENSIS*, *C. GIGAS* AND *C. SIKAMEA*. **Elizabeth A. Francis, Patrick M. Gaffney, Standish K. Allen,** and **Kimberly S. Reece,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062. E-mail: lizbeth@vims.edu

Despite the recent interest in marketing non-native oysters, little has been published about the distribution or population genetic structure of the Asian oyster *Crassostrea ariakensis*. Furthermore, morphological plasticity and hybridization with congeneric species *C. sikamea* and *C. gigas* make identifying this species difficult. Previously developed molecular typing keys produced conflicting results when typing individuals collected from sites in Japan and The Peoples Republic of China (PRC). The objectives of this project are to: (1) develop a reliable molecular typing key for distinguishing between sympatric Asian oysters; and (2) uncover the population structure of *C. ariakensis*.

Development of a molecular key distinguishing these species is underway using restriction length polymorphism analysis (RFLP) for both nuclear and mitochondrial loci. The ability of this key to distinguish between putative species is corroborated by the phylogenies produced from sequence data collected from both the nuclear ITS-1 locus and a fragment amplified from the mitochondrial CO1 locus. PCR products were amplified, cloned, and sequenced from at least two individuals of putative *C. ariakensis* representing each sampling site as well as individuals of *C. gigas*, *C. sikamea*, *C. belcheri*, and *C. virginica*. Phylogenetic analysis distinguished two closely related clades representing possibly two subspecies of *C. ariakensis*. One clade included the putative *C. ariakensis* individuals from Japan, northern China, and hatchery stocks; whereas, another group included most of the other samples from PRC. Individuals collected from Yangjiang County, Guangdong Province, PRC as putative *C. ariakensis* grouped with the *C. sikamea*. Phylogenetic results suggest that there is intraspecific variation within *C. ariakensis* that was not detected when the previous keys were developed. Sequence data were used to perform virtual digests from which to base the development of the key. Variation could be present in restriction sites thought to be

diagnostic for a particular species. This indicates the importance of determining the level of variation present within *C. ariakensis*.

Additional putative *C. ariakensis* samples and sympatric oyster species are being collected and analyzed from India and Vietnam. A genetic population survey shall be carried out for all individuals typing as *C. ariakensis*. Individuals (approximately fifty/site) will be subjected to PCR amplification of multiple markers, which will be digested with restriction enzymes cutting at polymorphic sites. Population genetic structure will be estimated by calculating genotypic and allelic frequencies.

IN SITU MONITORING OF PUMPING RATES IN THE OYSTER *CRASSOSTREA VIRGINICA* TO INVESTIGATE RESPONSE TO ENVIRONMENTAL CHANGE. Dana M. Frank and **J. Evan Ward,** University of Connecticut, Department of Marine Sciences, 1084 Shennecossett Road, Groton, CT 06340, USA.

One primary goal toward understanding compensatory responses of bivalve mollusks to environmental change is to obtain *in situ* measurements of physiologically relevant parameters. Feeding activity and its constituent components (e.g., pumping and clearance rates) are prime indicators of physiological response to environmental change. Although previous researchers have attempted to obtain field measurements since the late 1950s, at this time, no convenient method exists for the continuous monitoring of feeding activity of individual bivalves. Although controlled experimental manipulations have yielded valuable results, it is difficult to reproduce in the laboratory all the complex changes in environmental conditions to which animals are subjected in their natural habitats.

We have developed an optical biomonitor for recording *in situ* measurements of pumping pressures, rates, and valve gape in bivalves. Fiber optic sensors are used to measure alterations in both pressure and valve gape. The pressure sensor is situated in the suprabranchial chamber of the oyster. The valve gape sensor is attached to the right valve during experimental trials. With this arrangement, we can examine the relationship between valve gape and changes in pumping pressures to explore more thoroughly the mechanisms available to the bivalve for altering pumping rates. Results of preliminary laboratory trials have established the value of this system for recording changes in oyster pumping and valve gape continuously, both in the laboratory and *in situ*. By monitoring changes in activities over time, we hope to expand the scope of our understanding about the physiological responses of bivalves to changes in environmental parameters, such as temperature, dissolved oxygen concentrations, current velocity, and food availability.

TRIPLOID GIGANTISM IN MOLLUSKS AND POSSIBLE EXPLANATIONS. Ximing Guo, Zhaoping Wang, Standish K. Allen, Jr., and **Brenda J. Landau,** Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349, USA. E-mail: xguo@hsrl.rutgers.edu

Triploids are organisms with three sets of chromosomes instead of two sets in normal diploids. During the past two decades, triploids have been studied in over 20 species of mollusks. A review of available data indicates that superior growth or increased body size is a general feature of triploid mollusks. Triploids are significantly larger than diploids in almost all species studied so far. Triploids are observed to be larger than diploids by 12 to 30% in *Crassostrea virginica*, 25 to 51% in *Crassostrea gigas*, 42 to 52% in *Crassostrea dalienwhanensis*, 60% in *Ostrea edulis*, 41% in *Saccostrea commercialis*, 72% in *Mulinia lateralis*, 27 to 58% in *Pinctada martensii*, 36% in *Argopecten irradians*, 32 to 59% in *Chlamys nobilis*, and 81% in *Chlamys farreri*. Adductor muscles of triploid scallops are larger that that of diploids, by 73% in *A. irradians*, 44 to 96% in *C. farreri*, and 167% in *Argopecten ventricosus*. Guo and Allen (*Genetics* 1994, 138:1199–1206) proposed the concept of "polyploid gigantism" and attributed the increased body size in triploids as a function of increased cell size. Two other hypotheses have also been used in discussions on triploid mollusks: (1) the heterozygosity hypothesis that attributes the superior growth to increased heterozygosity in triploids; and (2) the sterility hypothesis that views that sterility in triploids results in energy relocation from reproduction to somatic growth. We considered all three hypotheses in an analysis of growth and genetics data from several types of triploids. Our analysis suggests that increased cell size is the fundamental cause for triploid gigantism in mollusks. The expression of triploid gigantism in meiosis II triploids may be limited by genetic problems from the retention of polar body II. Increased heterozygosity in mated (and sometimes meiosis I) triploids corrects genetic problems and permits efficient expression of triploid gigantism. Sterility or energy relocation helps to accelerate the expression of triploid gigantism in old animals. Triploid gigantism is manifest in certain organs, such as the adductor muscles of scallops and oysters. Triploid gigantism may not be expression in nutrient-limiting environments.

CONSUMER ATTITUDES AND PREFERENCES FOR OYSTERS: GULF OYSTER INDUSTRY PROGRAM. Terrill R. Hanson, Lisa O. House, and **Benedict Posadas,** Mississippi State University, Department of Agricultural Economics, P.O. Box 5187, Mississippi State, MS 39762. E-mail: hanson@agecon.msstate.edu

Although Gulf of Mexico oyster landings have remained fairly constant at 17.3 million pounds during the 1980s and 18.0 million pounds during the 1990s, average landings for the United States have decreased. There has been an 11.1 million pound decrease in

total U.S. oyster landings between the two decades with most of the decrease having occurred in eastern U.S. landings. To maintain or increase consumption of Gulf oysters, it is essential to determine consumer and nonconsumer preferences and consumption patterns, which will be important in strengthening the Gulf oyster industry. This project will determine oyster consumer demographics, consumption patterns, attitudes, and preferences allowing market segments to be identified and strategies to reach these audiences.

Specific objectives of this project are to: investigate the characteristics that influence regional variations in the consumption of oysters; evaluate the effects of oyster supply upon consumption; evaluate the effects of hazard analysis critical control point (HACCP)-based seafood inspection on oyster consumption; develop criteria for potential market segments; and disseminate information obtained from this research to appropriate processors, associations, and other audiences through publications and meetings.

Questionnaire development is centered on measuring consumers' preferences and attitudes about oyster purchase and consumption and developing demographics profiles for use in future marketing plans. Because many seafood consumers do not eat oysters, both consumers and nonconsumers of oysters will be surveyed. The specific configuration of the survey will be developed through exploratory research based on focus group methods. However, to obtain quantitative estimates, a "double hurdle" econometric model will be used. First, factors that affect the decision to consume oysters will be modeled. The results from this section will be important in determining the market segments that can be successfully targeted. Second, factors that affect the decision as to the quantity of oysters consumed will be studied. This will only apply to the subgroup of respondents that do consume oysters. Consumer attitudes toward food safety, perceptions of food safety for various oyster product forms compared with other meats, and consumer awareness of HACCP will be important explanatory variables. The marginal effects of these variables will allow the researchers to estimate the change in the probability of consumption of oysters because of HACCP programs.

Results from the survey and model will be used to estimate the effect of socioeconomic characteristics of consumers on demand for oysters. This information will then be used to determine viable market segments and to determine the best marketing efforts to reach those segments successfully.

MOLECULAR DIAGNOSTICS FOR THE PARASITIC DINOFLAGELLATE *HEMATODINIUM PEREZI*. **Karen L. Hudson, Jeffrey D. Shields,** and **Kimberly S. Reece,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062, USA. E-mail: khudson@vims.edu

Hematodinium species are parasitic dinoflagellates that infect a variety of marine crustaceans including crabs, lobsters, and amphipods. Outbreaks of this parasite proliferate in the hemolymph and cause excessive host mortalities. Species of *Hematodinium* and *Hematodinium*-like parasites have impacted commercial fisheries around the world. *Hematodinium perezi* has caused a decline in the commercially important blue crab (*Callinectes sapidus*) fishery in U.S. coastal areas from Delaware to Florida. Our primary goal in this study was to develop species-specific PCR primers and DNA probes to detect *Hematodinium perezi* infections.

To assess intraspecific DNA sequence variation within *H. perezi* strains we amplified and sequenced the internal transcribed spacer region (ITS) from two heavily infected crabs. Sequence data were then compared to previously published sequences. Although DNA clones from the heavily infected samples had insert sequences that showed high similarity to published *H. perezi* ITS sequences, several polymorphic nucleotide sites were identified.

To specifically amplify *H. perezi* small subunit (SSU) ribosomal RNA gene from host tissue, we utilized two sets of primers. The first set of primers was constructed from a conserved region of the SSU gene and from a previously published *H. perezi* partial SSU sequence. A reverse complement of the later primer was used in conjunction with a primer we designed from the ITS sequence regions exhibiting *H. perezi* specificity. We then aligned the SSU gene sequence of *H. perezi* with that of other dinoflagellates, host species, ciliates, and apicomplexans. Based on sequence comparisons we have designed several potential DNA probes for *in situ* hybridization of paraffin embedded infected host tissue sections. We are testing the probes against infected *C. sapidus*, *Necora puber* (velvet crab), *Chionoecetes bairdi* (tanner crab), and *Nephrops norvegicus* (Norway lobster), in hopes of identifying both species- and genus-specific DNA probes. We are also using the SSU sequence data to elucidate the taxonomic relations within the genus.

A SYSTEMATIC APPROACH TO DEVELOP AN OYSTER CELL LINE. **Jerome La Peyre, Yanli Li, John Buchanan, Philip Cheng, Richard Cooper,** and **Terrence Tiersch,** Department of Veterinary Science, Louisiana State University, Baton Rouge, LA, 70803, USA. E-mail: jlapeyre@agctr.lsu.edu

No oyster cell line has been developed despite numerous attempts over the last fifty years. A major difficulty is that the proliferation of oyster cells cannot be maintained *in vitro*. Moreover, although numerous procedures have been used to prepare primary oyster cell cultures, the viability and survival time of oyster cells obtained from dissociated tissues have been disappointing.

Our working hypothesis is that the lack of proliferation is mainly attributable to suboptimal techniques and conditions that have been used to establish and maintain oyster cells in culture. Therefore, a systematic approach is needed, not only to optimize procedures to establish primary cultures, but also to optimize culture media and physical conditions to maintain viable oyster cells

over an extended period of time (i.e., weeks, months) a prerequisite to developing a cell line.

During the past several years, we have optimized techniques to decontaminate and dissociate tissues of the eastern oyster *Crassostrea virginica* and to cryopreserve oyster cells. A culture medium was formulated and optimized. Attachment factors to improve cell adherence and spreading were also tested. As a result, cell survival was significantly increased by optimization of these procedures. In this presentation, we review some of the problems encountered in the culture of eastern oyster cells and discuss additional strategies that are being used to develop an oyster cell line.

FORMULATION AND OPTIMIZATION OF A CULTURE MEDIUM FOR CELLS OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*. **Jerome La Peyre, Yanli Li,** and **John Buchanan,** Department of Veterinary Science, Louisiana State University, Baton Rouge, LA 70803, USA. E-mail: jlapeyre@agctr.lsu.edu

The optimization of a culture medium for oyster cells may be a critical factor for the development of a cell line. A variety of commercial culture media supplemented with fetal bovine serum have been used to maintain oyster cells in primary cultures. Although certain ingredients found in oyster plasma are often added to these commercial media, there have been limited attempts to evaluate the benefits of these ingredients to oyster cells and to optimize their concentrations. Moreover, no culture medium has yet been formulated specifically for cells of oysters or other bivalve mollusks. Therefore, a strategy was adopted to develop a culture medium for oyster cells methodically.

A basal medium composed of a mixture of inorganic salts, buffers, amino acids, carbohydrates, and vitamins that resembled the known composition of oyster plasma was initially prepared. The concentrations of each mixture were optimized by comparing the metabolic (dehydrogenase) activity of heart cells, as measured by the reduction of tetrazolium salts to formazan, and by observing the morphology and contractility of heart cells in culture. Our basal medium, designated LA-1, was found to be superior to L-15 medium, a commercial medium that has most often been used for oyster cells.

Using our basal medium LA-1, the effects of more than 30 ingredients on primary heart cell cultures were then evaluated individually over a broad range of concentrations and in combination using a statistical optimization approach based on a Plackett–Burmann design. A defined medium, designated LA-2, was formulated by supplementing our basal medium with all beneficial ingredients. LA-2 was far better than L-15 supplemented with fetal bovine serum (FBS) for maintaining oyster cells in primary cultures. Moreover, addition of low concentrations of FBS (i.e., 2 and 4%) to LA-2 did not benefit oyster heart cells; whereas, the addition of higher concentrations of FBS (i.e., 6 and 8%) to LA-2 was detrimental.

Finally, we tested a number of undefined medium supplements (e.g., lactalbumin hydrolysate, egg yolk) reported to be beneficial to oyster cells as well as sera from a variety of animals (e.g., goat, chicken, fish). Their effects on oyster heart cells maintained in LA-2 were determined individually and in combination to select beneficial supplements. The culture medium developed greatly increased the survival and metabolic activity of oyster cells *in vitro* as compared to L-15. This medium will be used to stimulate cell proliferation with known mitogens in future studies.

EFFECTS OF OYSTER REEFS ON MICROBIAL COMMUNITY STRUCTURE AND PRODUCTION. **Alan J. Lewitus, Michael S. Wetz, Eric T. Koepfler, Kenneth C. Hayes,** and **Richard F. Dame,** Baruch Marine Field Laboratory, Baruch Institute, University of South Carolina, P.O. Box 1630, Georgetown, SC 29442, USA. E-mail: Lewitus@belle.baruch.sc.edu

Oyster reefs can influence microbial communities through grazing and nutrient regenerative activities. Each process can be selective. Therefore, the net effect of oyster reefs on microbial food webs is a function of the combined influences of oyster reef feeding preference and microbial uptake ability for regenerated nutrients (e.g., NH_4). We compared: (1) microbial community composition and biomass in intertidal creeks with (+Oys) and without (−Oys) oyster reefs; (2) the potential for nutrient or grazing limitation of phytoplankton from those creeks using bioassays; and (3) changes in microbial communities in water flowing across oysters in linear flumes. When comparing all +Oys to −Oys creeks, no significant differences in NH_4 or chlorophyll *a* (chl) were found, nor did the responses of chl to nutrient additions or reduced grazing pressure differ in bioassays. However, when geomorphologically similar +Oys and −Oys creeks were paired, differences in NH_4, chl, and bioassay responses were evident, suggesting that the influence of oyster reefs on phytoplankton growth and nutrient availability depends on creek geomorphology. A consistent effect of oyster reefs on phytoplankton composition was seen in summer, when +Oys creeks contained significantly fewer phototrophic flagellates, but not heterotrophic flagellates, ciliates, bacteria, cyanobacteria, or diatoms. Flume experiments demonstrated preferential removal of phototrophic flagellates, but not other groups, by live oysters but not dead oyster shells. Thus, oyster reefs can exert control over microbial food web structure, but their impact on microbial community production apparently depends on tidal creek morphology and hydrography. When examined across a range of creek types, the influence of oyster reefs on microbial community composition and nutrient availability did not affect over-all nutrient concentration or phytoplankton biomass, suggesting that other grazers (e.g., zooplankton, nekton) or nutrient sources (e.g., zooplankton, nekton, groundwater) may override or compensate for the effects of oyster reefs.

EVALUATING POTENTIAL COMPETITIVE INTERACTIONS BETWEEN *CRASSOSTREA VIRGINICA* AND NONINDIGENOUS OYSTER SPECIES IN THE CHESAPEAKE BAY. **Mark W. Luckenbach,** and **Albert G. Curry, Jr.,** Virginia Institute of Marine Science, College of William and Mary, Wachapreague, VA, 23480, USA. E-mail: luck@vims.edu

Declines in standing stocks of native oysters, *Crassostrea virginica*, throughout the mid-Atlantic coast of the United States has focused attention on management options for using nonindigenous species for the purposes of enhancing wild fisheries, reestablishing ecosystem services, and promoting aquaculture development. Recently, small-scale commercial trials using triploids of the exotic oyster species *Crassostrea ariakensis* have been permitted in Virginia. Despite the sterility of triploids, this practice presents some risk of unintended reproduction and, thus, introduction of the exotic species. To evaluate both the utility of nonindigenous species for these purposes and the risks posed by their introduction, we have conducted a series of quarantined laboratory and field experiments using sterile triploids of the Pacific oyster species *C. gigas* and *C. ariakensis*.

Among the issues of concern posed by any such introduction is the outcome of potential competition between native and nonnative species. In a series of quarantined, flow-through laboratory flume experiments, we have investigated competitive interactions between *C. virginica* and either *C. gigas* or *C. ariakensis*. Replicate treatments consisting of the native and one non-native oyster species in five proportions (100:0, 90:10, 50:50, 10:90, 0:100) were established with newly settled juvenile oysters onto ceramic tile plates. These plates were photographed, and the location of each individual of each species was mapped with an image analysis system. Plates were then placed individually in an array of flow-through pipe flumes. Effluent from the flumes was filtered through a series of sand filters before discharge. On a weekly basis for 10 to 12 weeks the plates were removed, gently rinsed, and photographed in a standard orientation. Image analysis was used to track growth, survival, and the outcome of intra- and interspecific interactions. The results indicate complex patterns of species interactions dependent in part upon the relative orientations of individuals. Moreover, they point to the uncertainty associated with predicting the ecological risks associated with the introduction of a non-native oyster and provide a cautionary note to fisheries managers considering such an option.

PHOSPHOLIPID SYNTHESIS IN PERKINSUS MARINUS: A PRELIMINARY INVESTIGATION OF FACTORS LIMITING CELL REPLICATION. **Eric Lund, Philippe Soudant,** and **Fu-Lin E. Chu,** Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, P.O. Box 1346, 1208 Greate Road, Gloucester Point, VA 23062, USA. E-mail: lund@vims.edu

Perkinsus marinus is a protozoan parasite that causes high mortality rates in its commercially important host, the eastern oyster *Crassostrea virginica*. Previous studies in our laboratory and elsewhere have shown that amino acids in the media were almost completely depleted when meront cultures of *P. marinus* reached stationary phase. We hypothesize that the termination of cell proliferation may be due to the depletion of polar headgroups in the media, not the ability of the parasite to synthesize polar lipids *de novo*. To determine the phospholipid nutritional requirements of *P. marinus*, we investigated the ability of this parasite to synthesize phospholipids from various polar headgroup precursors. Pulse/chase experiments using precursors of polar lipid headgroups revealed that *P. marinus* meronts rapidly utilized exogenous amino acids and sugars for incorporation in a wide variety of polar lipids (Table 1). Specifically, serine was incorporated into phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and ceramide (CER). Ethanolamine was incorporated into PE and PS. Choline was incorporated into PC. Inositol was incorporated into phosphatidylinositol (PI) and other glycolipids. Preliminary experiments also revealed that cell replication occurred in lipid-free media containing amino acid mixtures and other nutrients. Cell numbers increased from 0.8×10^6 cells mL^{-1} to 1.59×10^6 cells mL^{-1} in seven days. These results suggest that *P. marinus* may not require intact polar lipids from its host for membrane synthesis and proliferation.

TABLE 1.

Activity recovered (nCi) in HPTLC scrapings of extracted culture lipids after incubation of 2×10^6 *P. marinus* meronts with 15 µCi of a single radiolabeled headgroup in 5 ml 0.22 µm filtered York River water (28 ppt) containing 10 µg ml^{-1} cod liver oil for 2 hr.

Headgroup	PC	PS and PI	PE*	CER and unknown polar lipids
^{14}C choline	40.65	0.45	0.08	0.18
^{14}C ethanolamine	0.03	0.10	1.29	0.55
^{3}H serine	8.65	17.44	39.95	27.17
^{3}H myo-inositol	0.10	2.56	1.90	1.23

* This fraction may also contain unidentified glycolipids.

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ALTERED *PERKINSUS MARINUS* PROTEASE PROFILES UPON EXPOSURE TO SELECTED OYSTER TISSUE HOMOGENATES. **Alanna MacIntyre** and **Stephen Kaattari,** Department of Environmental Sciences, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA, USA. E-mail: alanna@vims.edu

Utilizing *in vitro* culture methodologies for the growth of *P. marinus*, we have examined the possible role of oyster tissue factors in the modification and/or regulation of parasitic secretory products. Various concentrations of oyster tissue homogenates were used to supplement a defined culture medium. The growth

rate and protease expression with the various supplements was then determined. It was found that although the growth rate of the *P. marinus* cells is relatively unchanged upon exposure to the homogenates of various oyster species, a distinctly different array of proteases, as determined by gelatin-gel electrophoresis were observed with *Crassostrea virginica* homogenates, but not with *Crassostrea ariakensis*. Typically at least five proteases within the molecular weight range of 65–135 kDa can be observed in bovine serum albumin (BSA)-free medium or BSA-free medium supplemented with *C. ariakensis* or homogenates. However addition of *C. virginica* homogenates reveals the presence of five additional protease bands in the 10–50 kDa molecular weight range. This is of particular interest, because *C. virginica* is the most susceptible oyster species to *P. marinus* infection. This research was funded by the NOAA–Virginia Sea Grant Association, Oyster Disease Research Program.

TEMPORAL VARIATION IN THE GAMETOGENIC CYCLE OF MARINE MUSSELS, *MYTILUS EDULIS* AND *MYTILUS TROSSULUS*, IN COBSCOOK BAY, MAINE. **Aaron Maloy, Bruce J. Barber,** and **Paul D. Rawson,** University of Maine, School of Marine Science, 5735 Hitchner Hall, Orono, ME 04469-0001. E-mail: aaron.maloy@umit.maine.edu

Culture of the edible blue mussel, *Mytilus edulis*, is expanding in the state of Maine to meet a growing demand. Current practice involves the dredging of juveniles from natural seed beds for placement on bottom leases or collection of seed on ropes for suspension from rafts. It has recently been discovered that a second species of mussel, *Mytilus trossulus*, occurs sympatrically with *M. edulis* in eastern Maine. Future economic viability of the industry is dependent on the ability of growers to collect the more valuable *M. edulis* seed preferentially. A lack of natural hybridization between the two species and observations of two distinctive spat settlements in the region suggests there may be a temporal difference in spawning that could be utilized for preferential collection of *M. edulis* seed. This study was undertaken to compare the gametogenic cycles of *M. edulis* and *M. trossulus* from a single population from Cobscook Bay, Maine.

Throughout 2000, one hundred to one hundred thirty mussels were sampled from a low intertidal site at monthly to bimonthly intervals. Three-polymerase chain reaction (PCR)-based nuclear markers and one ribosomal marker were used to differentiate between mussels species. Histologically based quantitative assessments of gametogenesis were carried out using image analysis software. The gametic volume fraction (GVF) for twenty individuals of each species was obtained from each sample and used to make assessments concerning gametogenic development and timing of the spawning events. Preliminary analysis indicates that *M. trossulus* initiates gametogenesis earlier than *M. edulis*.

MASS MORTALITIES OF SOFT-SHELL CLAMS (*MYA ARENARIA*) IN ATLANTIC CANADA ASSOCIATED WITH UNPRECEDENTED LEVELS OF HEMIC NEOPLASIA. **Sharon E. McGladdery,[1] Gregory S. MacCallum,[2] Neil G. MacNair,[3]** and **Jeffrey T. Davidson[2],** [1]Department of Fisheries and Oceans, Canada, Gulf Fisheries Centre, Moncton, NB, Canada, E-mail: mcgladderys@dfo-mpo.gc.ca; [2]Atlantic Veterinary College, UPEI, Charlottetown, PEI, Canada; and [3]PEI Department of Fisheries and Environment, Charlottetown, PEI, Canada.

In July, 1999, a soft-shell clam (*Mya arenaria*) farmer reported heavy losses that seemed to be increasing in severity. A sample of moribund and neighboring, apparently healthy, clams showed levels of hemic neoplasia >95%. Low prevalences (<13%) of hemic neoplasia has been reported previously from soft-shell clams in Atlantic Canada (Morrison *et al.* 1993) and over 2,000 soft-shell clams sampled between 1990 and 1999 revealed equally low sporadic prevalences (<11%, unpublished data). This is the first case of mortalities being associated with the condition in Atlantic Canada.

Repeat samples of different size groups in August found 20 to 60% in one- to five-year-old specimens. Archived samples from reproductive studies by the Department of Fisheries and oceans (DFO) St. Andrews in the Bay of Fundy, showed prevalences <10% between 1989 and 1991. Similar samples held at the Atlantic Veterinary College showed prevalences of 0 to 20% in 1997. In 1998, samples from a neighboring site showed an over-all prevalence of 5%. This site showed an increase to 8% in June 1999 and 80% in August, with associated mortalities.

A broad-scale survey of PEI clam beds revealed negative and positive sites with linkages to seed collection and transfers, as well as in close proximity to agriculture activity; thus, the cause of the neoplasia increase is not clearly evident. Interestingly, an independent submission from New Brunswick revealed a similar high prevalence of neoplasia in weak and dying clams (34%), indicating a possibility that this may be wider spread than in PEI. It also suggests that warm, shallow Gulf of St. Lawrence waters may be a contributing factor.

To date, only soft-shell clams are affected, which is consistent with similar mortalities reported from the eastern United States (Barber 1990, Farley et al. 1991). Transmission experiments are currently underway to determine whether or not this is a transmissible condition or more closely related to environmental triggers.

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THE USE OF *CRASSOSTREA VIRGINICA* CULTCH TO STABILIZE AND ENHANCE CREATED *SPARTINA ALTERNIFORA* MARSH. David L. Meyer, NOAA Beaufort Laboratory,101 Pivers Island Road, Beaufort, NC 28516, USA. E-mail: dave.meyer@noaa.gov

In 1987, three dredge material sites were sculpted and planted with *Spartina alterniflora*. By spring 1992, the plantings had completely colonized the sites. These sites were modified in 1992 by the addition of *Crassostrea virginica* cultch to the lower intertidal fringe of select portions of the created marshes. Twelve 5-m wide plots were established at each site, six randomly selected plots remained unaltered (noncultched), and six plots were modified by addition of 1.5-m wide, 4.5-m long, and 0.25-m deep band of cultch (cultched). This was performed to examine: (1) the cultch additions potential to stabilize marsh vegetation and sediment; (2) cultch use by oyster reef fauna; and (3) the effect of increased marsh habitat heterogeneity, via cultch addition, on nekton.

Marsh edge vegetation stability and sediment erosion was measured for each plot from September 1992 to April 1994. A significant difference ($P < 0.05$) in marsh edge vegetation change was detected at the only south facing site after a major southwester storm. Significantly different rates of sediment erosion (noncultched plots lost 3.2 cm) and accretion (cultched plots accreted 6.3 cm) also occurred. A second site, with a northern orientation, also experienced differential sediment accretion and erosion between plot type. Here, the results were in response to boat wakes that were magnified by the abutment of a dredge effluent pipe across the front of the site. During this time period, we observed sediment accretion in the cultched plots of 2.9 cm and erosion in the noncultched plots of 1.3 cm.

Cultch plots supported numerous oyster reef associated fauna at densities equivalent to nearby natural reefs. Two years after placement, harvestable sized *C. virginica* (> 75 mm) were present within the cultch. Cultched plots also contained a higher number of oyster-associated species than natural reefs and noncultched plots. After two years, densities within the cultched plots for the most abundant species including *C. virginica, Balanus amphitrite, Panopeus herbstii,* and *Eurypanopeus depressus* were equivalent to natural reefs. These species were not present within the noncultched plots. In addition, the fringe of oyster cultch seemed to enhance marsh use by some marsh resident nekton species and those known to have an affinity for oyster substrate, including *Cyprinodon variegatus, Fundulus heteroclitus, Gobionellus boleosoma, Gobiosoma bosci,* and *Palaemonetes* spp. The cultch may, however, serve as a barrier for species that move onto the marsh early in the tidal cycle including *Leiostomus xanthurus* and *Callinectes sapidus.*

Cultch placement at the lower fringe of created salt marsh can reduce marsh sediment erosion and vegetation loss in areas prone to storm-related wave activity. The addition of cultch to the lower marsh fringe may promote resident marsh nekton use beyond that of noncultched marsh. Furthermore, cultch additions can provide quality habitat for oyster reef fauna.

***IN VITRO* STUDIES OF DISSEMINATED NEOPLASMS OF *MYTILUS TROSSULUS*. James D. Moore, Hiroko Shike, Chisato Shimizu, Ralph A. Elston,** and **Jane C. Burns,** U.C. Davis Bodega Marine Laboratory, 2009 Westside Road, Bodega Bay, CA 94923, USA. E-mail: jimmoore@ucdavis.edu

Establishment of a cell line requires immortalized or transformed cells that divide continuously in culture. Naturally occurring neoplasms provide a source of highly concentrated, rapidly proliferating, transformed cells. The only impediment to establishing a cell line from this material is to identify culture conditions that will support long-term growth. We are exploring the *in vitro* culture of disseminated neoplasms of mussels, *Mytilus trossulus* from Puget Sound, Washington, U.S.A. Approximately 0.5–1% of mussels in these populations contain advanced neoplasms in which more than 90% of the cells in the hemolymph are rapidly cycling, transformed cells with altered DNA content.

We assessed the cycling activity of normal and neoplastic cells *in vitro* by tritiated thymidine incorporation. Cells from a mussel with an advanced neoplasm showed different levels of DNA synthesis in different media, thus demonstrating the utility of this assay for optimizing culture conditions.

To study the transmissibility of the neoplasms, we sought a method to mark the cells genetically. Neoplastic cells were exposed *in vitro* to pantropic retroviral vectors that contain a substituted envelope protein that permits infection of invertebrate cells. The vector, LLRNL (Moloney murine leukemia virus long terminal repeat (LTR) (L)-firefly luciferase (L)-Rous sarcoma virus LTR (R)-neomycin phosphotransferase (N)-MoMLV LTR), was used to infect cells. Transduction of cells was stable over a six-day period, was independent of polybrene, and was proportional to number of vector particles. These results suggest that pantropic vectors can be used to mark genetically neoplastic cells *ex vivo*, which can then be transplanted into healthy mussels to study transmission and provide a continuous source of neoplastic cells for *in vitro* experiments.

OPTIMIZING CULTURE CONDITIONS FOR CREATION OF AN OYSTER CELL LINE. James D. Moore, Viviane Boulo, Chisato Shimizu, Hiroko Shike, Carolyn S. Friedman, and **Jane C. Burns,** U.C. Davis Bodega Marine Laboratory, 2099 Westside Road, Bodega Bay, CA 94923, USA. E-mail: jimmoore@ucdavis.edu

To aid in the formulation of media to support oyster cells nutritionally in culture, we analyzed the hemolymph components from *Crassostrea virginica* and *C. gigas*. We measured DNA synthesis in primary cultures incubated in different media and at different temperatures to optimize culture conditions.

Pooled hemolymph (two to three oysters/pool) was obtained by cardiac puncture. Hemocytes were removed by centrifugation (1,000 × g) and the supernatants stored at −70°C. Analysis of hemolymph components included free amino acids, organic acids, carbohydrates, metals, electrolytes, pH, and osmolality. Cultures of heart and embryos were established according to published methods (Boulo et al. 1996, 2000). DNA synthesis in cultured cells was assessed by ^{3}H-thymidine uptake.

Two media formulations based on published data were compared: (1) KS medium (based on Kleinschuster and Swink 1993): L-15 adjusted to 750 mOsm with synthetic sea salts, and supplemented with amino acids, lipids, carbohydrates, vitamins, 10% fetal calf serum (FCS), and 10% *C. virginica* hemolymph; (2) 2X L-15 (Boulo et al. 1996) adjusted to 750 mOsm with NaCl plus 10% FCS.

Selected components that were lower in the media than in the pooled hemolymph samples are shown below (mean values ± SD; NA = not available)

Component	*C. virginica* hemolymph	KS medium	2X L-15 medium/10% FBS
Taurine	195.9 ± 12.1mg/L	51.9	NA
Proline	90.5 ± 3.7	34.2	NA
Calcium	40.7 ± 6.2	19.8	9.3
Strontium	4.7 ± 0.9	3.0	0.1
Boron	4.4 ± 0.7	NA	0.1
Zinc	1.3 ± 1.3	NA	0.1

pH ranged from 6.4–6.9.

Preliminary comparison of ^{3}H-thymidine uptake by primary *C. gigas* heart cells cultured in different media at different temperatures revealed that DNA synthesis occurred between 48 and 72 h postinitiation of the cultures under all conditions. In contrast, dissociated embryo cells had maximal DNA synthesis during the first 24-h period in culture and 2X L-15 at 23°C supported the highest level of DNA synthesis. Additional comparisons will guide formulation of media to nutritionally support primary cultured oyster cells. Supported in part by California Sea Grant NA86RG0073 Project No. R/A-110B and the California Department of Fish and Game.

REVIEW OF GULF OYSTER INDUSTRY PROGRAM GRANT PROJECTS: LOUISIANA OYSTER LEASES VERSUS COASTAL RESTORATION AND CLEAN-UP OF CONTAMINATED OYSTER BEDS. Erinn W. Neyrey, Joe Stevenson, and **Michelle Marney,** LSU Sea Grant Legal Program, LSU Law Center, Rm. 170, Baton Rouge, LA 70803, USA. E-mail: eneyrey@lsu.edu

Louisiana oyster leases versus coastal restoration: Louisiana's coastal wetlands account for more than 25% of all wetlands found within the United States; however, this important ecosystem is disappearing at an astonishing rate. To counter coastal erosion, Louisiana has developed an extensive plan consisting of numerous restoration projects. One of the major components of the plan is the construction of freshwater diversion projects. The diversion of sediment-rich river water is designed to mimic the natural flood cycle and allow the river's sediments and nutrients to be deposited in the coastal zone. Oyster farmers in the restoration areas have experienced negative impacts on their over-all productivity caused by the changes in salinity. These impacts have led to lawsuits being filed by the oyster farmers against the state for damages. In response, Louisiana has passed legislation creating the Oyster Lease Relocation Program (OLRP). The OLRP is designed to offer alternatives to the oyster farmers who find themselves in a restoration area. Although this program may not provide a cure for all of the problems that may arise, it does provide an extrajudicial means to resolve many of the disputes between oyster farmers and the state resulting from coastal restoration projects.

Clean-up of contaminated oyster beds: Louisiana is the top processor of oysters within the Gulf region, which produces nearly 60% of the nation's oysters, and is home to nine hundred oyster farmers. Successful business planning by members of this significant industry benefits not only those in the industry, but the state as well. Relocation of leases because of fouled oyster beds effects not only the livelihood of an oyster farmer, but lifestyle as well. Both the Clean Water Act and Louisiana's Water Control Law clearly prohibit the discharge of pollutants into waters; however, these laws do little to address the actual clean-up of polluted water bodies. Therefore, solutions to the water pollution problems are turning increasingly on interpretive regulation and policy of the U.S. Environmental Protection Agency and the private citizen actions that seek alternatives to the imposition of civil penalties. Supplement environmental projects offer a potential flexibility that traditional enforcement actions do not.

A REVIEW OF ORGANISMS ASSOCIATED WITH OYSTERS CULTURED IN FLOATING SYSTEMS. F. X. O'Beirn, P. G. Ross, and **M. W. Luckenbach,** Virginia Institute of Marine Science. E-mail: francis@vims.edu

Given the increase in oyster (*Crassostrea virginica*) aquaculture on the eastern seaboard of the United States, an issue to be considered is the influence of these intensive operations on faunal assemblages found adjacent to and among the oysters themselves. In fact, little is known concerning the organisms associated with such aquaculture operations. Using oysters in a typical culture scenario, we assessed the number of macrofaunal species associated with floating culture systems and the number of organisms within broad taxonomic groups. Within each floating structure (2 ft × 8 ft × 1 ft) oyster numbers ranged from 488 to 1,381. Overall, forty-four species of macrofauna were recorded from the floating structures. The species richness within each float ranged from twenty-four tothirty-six. Abundances ranged from 12,746 to 92,602 individuals per float. In an attempt to determine if the organisms were influenced directly by live oysters or the structure

provided by oyster shell, we evaluated communities associated with live oysters, oyster shell, and empty floats (culture systems). Preliminary results indicate that the live oyster and oyster shell have similar abundance and biomass of individuals, but the composition of individuals is different. These results suggest that structure of any sort is important to certain species (e.g., grass shrimp), but the presence of live oysters influences the presence of other species (e.g., anemones, blue mussels). Given the ephemeral nature of these communities (mediated by harvest and handling schedules), many organisms within these floating communities may not actually mature to reproduce, and these systems may be sink populations. Certain associated species may actually compete with the oysters for food resources (e.g., blue mussels) and increase the organic loading emanating from the culture systems. In addition, altering the habitat type in a particular area may change the constituent organisms of a community and species interactions within a system. For example, fishes nesting or taking refuge in the cages may reduce grazer abundances (e.g., amphipods); thereby, affecting macroalgal abundances. As the scale of aquaculture operations increase, it will be increasingly important to further elucidate these and other ecological interactions.

SPATIAL AND INTERANNUAL OCCURRENCE OF A BROWN SHELL CONDITION IN NEWFOUNDLAND FARMED BLUE MUSSELS (*MYTILUS* SPP.) G. Jay Parsons, Kelly Moret, Cyr Couturier, and **Miranda Pryor,** Centre for Aquaculture & Seafood Development, Marine Institute, Memorial University, P. O. Box 4920, St. Johns, NF, Canada A1C 5R3. E-mail: Jay.Parsons@mi.mun.ca

The appearance of a brown coloration on the surface of blue mussels in Newfoundland prompted an investigation into the extent, potential impact on mussels, and factors influencing its occurrence throughout the province. In severe infections, this brown shell condition results in the loss of the periostracum and has been described as the "mycotic periostractal sloughing" (MPS) disorder. The etiological agent is a fungus yet to be identified (T. J. Davidson, pers. comm.); however, similar mycotic infections are common in cultured mussels worldwide. The mode of transmission is unknown, but studies on PEI mussels suggest that initial infection occurs in early fall in mussels that are more than a year old (T. J. Davidson, pers. comm.). The objectives of this study were to determine the spatial distribution of the fungal shell infections (FSI) (resulting in the brown shell condition) throughout Newfoundland, determine the interannual variability, and determine if progression is size related.

The shells of mussels were examined from twenty-one culture sites throughout the province in 1998 and twenty-two sites in 1999. There were about sixty mussels sampled from each site. Mussels ranged in size from about 35 to 85 mm shell length. Mussels were examined for the occurrence of FSI on the surface of the exterior shell using a dissecting microscope to observe the occurrence of brown foci. On mussels with FSI present, the percentage coverage (to the nearest 5%) was recorded. Data on the occurrence and percent coverage were summarized by farm site and geographic zone.

The occurrence of FSI in both years ranged from 0 to 100% of mussels at sites around Newfoundland. At sites where the brown shell condition was recorded, the percentage coverage ranged from 1 to 93% and 1 to 99% for 1998 and 1999, respectively. When the farms were grouped by zones, the most northern sites had the lowest occurrence of FSI and the lowest percentage coverage. Sites in central Newfoundland had a higher rate of occurrence but a low percentage coverage. Sites on the south coast had both a high rate of occurrence and a high percentage coverage of FSI. A similar pattern occurred in both years of the study. There were, however, no consistent differences in the occurrence or percentage coverage of FSI on individual sites from 1998 to 1999. Some sites had a lower rate of occurrence of FSI in 1998 as compared to 1999, but other sites had a higher rate in 1998 as compared to 1999. Percentage coverage was significantly, but weakly, correlated to mussel shell length for both 1998 and 1999. There was no apparent decrease in condition or health in mussels with the brown shell condition.

Mussels with FSI were found throughout Newfoundland; however, the condition known as MPS was rarely observed even in larger, older mussels. Although there were distinct geographic differences in the rate of occurrence and percentage coverage, we have not yet been able to relate these differences to specific environmental parameters or husbandry conditions, except that it seems size/age, temperature, and husbandry practices may influence the extent of coverage.

INDUCTION OF TRIPLOIDY IN THE AMERICAN OYSTER *CRASSOSTREA VIRGINICA*: A RE-EVALUATION OF POLAR BODY I INHIBITION. Stefano Peruzzi and **Ximing Guo,** Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349, USA. E-mail: speruzzi@hsrl.rutgers.edu

In mollusks, triploidy can be induced by inhibiting either polar body 1 (PB1) or polar body 2 (PB2). It has been shown in the Pacific oyster that PB1 inhibition results in primarily aneuploids and heavy larval mortality. In the American oyster, an early study has shown that PB1 triploids grow faster than diploids and PB2 triploids, possibly because PB1 triploids are more heterozygous. The objective of this study is to re-evaluate if PB1 inhibition is a valid method for triploid induction and if PB1 triploids grow significantly faster than diploids and PB2 triploids in New Jersey. Growth performance of triploids is known to vary among different environments.

In this work, three experimental groups were produced: a normal diploid control, a PB1 triploid group, and a PB2 triploid group. Cytochalasin B (0.75 mg/L) was used as the induction agent. The

experiments were replicated four times using a total of twenty-five females and fifteen males oysters. Both methods were effective in generating variable levels of triploidy (41 to 100%). Viability of PB1 triploids was actually higher than PB2 triploids over the first two months of life. Oysters will be deployed at two sites in NJ. Oysters will be sampled at regular intervals for ploidy determination and growth measurements. Genomic heterozygosity of experimental oysters will be determined using allozyme markers. Sampling and analyses are currently underway and will be reported at the meeting.

REDUCTION OF RED TIDE TOXIN IN CLAMS BY OZONE PURIFICATION AND RELAYING. **Richard Pierce, Michael Henry,** and **Gary E. Rodrick,** University of Florida, Department of Food Science and Human Nutrition, Gainesville, FL, USA. E-mail: GER@GNV.IFAS.UFL.EDU

A study of the accumulation and purification of harmful algal biotoxins in the clam, *Mercenaria mercenaria* was initiated to investigate the use of ozone as a means to enhance the natural purification of toxins. Clams were exposed to viable cells of the Florida red tide organism, *Gymnodinium breve* (5 × 106 cells/clam/day) for nine days. Following exposure, the clams were divided into groups for natural relay versus ozone purification. The concentration of brevetoxins (PbTx-2 and PbTx-3) in the exposure water and clam tissue was monitored by high-power liquid chromatography–ultraviolet (HPLC–UV) analysis and by receptor-binding assay. The amount of total PbTx-2 and PbTx-3 in the original culture before dilution for clam exposure and brevetoxin concentration in the effluent water after exposure clams show that the clams were exposed to appropriate levels during the study. These results show the presence of brevetoxins in the spiked samples and a definite accumulation of active toxins after ten days of exposure. After three days of ozone purification, the treated clams exhibited a 50% drop in the brevetoxin activity level; whereas, the nonozone-treated clams exhibited a 38% drop in the ten-day exposure level. Relaying the red tide contaminated clams to a clean seawater area for fifteen days removed (100%) toxin activity when compared to nonrelayed controls.

OYSTERS REEFS AS HABITAT FOR FISH AND DECAPODS: SPECIES AND LANDSCAPE CONSIDERATIONS. **Martin H. Posey, Troy D. Alphin,** and **Thomas K. Frazer,** Center for Marine Science and Department of Biological Sciences, University of North Carolina at Wilmington, One Marvin K. Moss Lane, Wilmington, NC 28409, USA. E-mail: Poseym@uncwil.edu

Structural habitats have long been recognized as important nursery areas for juvenile fish and decapods, as indicated by work in seagrass and marsh habitats. Recent interest has focused on the potential importance of oyster reefs as a refuge habitat. Along much of the southeastern coast of the United States and in some areas of the Gulf of Mexico, seagrasses are largely absent, and oyster reefs may provide the primary structural habitat in low to shallow subtidal areas. However, use of these oyster reefs may vary among species and may depend on the occurrence of other habitat types.

We have examined abundances of decapods and fish in oyster and marsh edge/sand habitats in two regions of North Carolina—one that has seagrasses (New River) and one that completely lacks seagrass beds (Hewletts Creek). In the absence of seagrass beds, grass shrimp and certain fish were more abundant within oyster beds during peak periods of abundance in winter and spring (Fig. 1). However, other decapods may not show this pattern. In the New River, where seagrasses provide an alternative habitat, there were low abundances of all groups sampled within oyster beds (Fig. 2). Abundances were somewhat higher in sand/marsh edge habitats and were much higher for most groups within seagrass beds. In all cases, there are strong seasonal effects on use of oyster beds, including shifts in the major species present.

Our results emphasize the potential importance of oyster reefs as habitat for certain fish and decapods. However, the patterns of use varied between locations (especially with the presence of alternative habitats), varied between taxa, and varied seasonally.

DERMOWATCH: A WEB-BASED APPROACH FOR MANAGING *PERKINSUS MARINUS* DISEASE OF OYSTERS. **Sammy M. Ray, Thomas M. Soniat,** and **Enrique V. Kortright,** Department of Biological Sciences, Nicholls State University, Thibodaux, LA 70310, USA. E-mail: biol-tms@mail.nich.edu

A web site called DermoWatch (www.blueblee.com/dermo) has been established to help manage *Perkinsus marinus* (= *Dermocystidium marinum*) disease of eastern oysters, *Crassostrea virginica*. The main page provides the most recent data for nine stations in Galveston Bay, Texas. Data include water temperature (T) and salinity (S), weighted incidence (WI) and percentage infection, and estimated time to a critical level of disease (t_{Crit}). Archived historical data for each site is available from the main page and via a map of the bay. With a utility called the Dermo Calculator, the web site is useful wherever dermo disease is found. The Dermo Calculator allows anyone with information on water T and S, oyster length (L), and initial WI of disease to calculate a t_{Crit}. A prototype utility has also been developed that uses real-time values for water T and S from a fixed monitoring station. The utility requires input of the time period of interest and L; it returns, in graphical form, a time course of T, S and t_{Crit}. More frequent

values of T and S permit more frequent estimates of t_{Crit} and should increase the reliability of the model.

INTER- AND INTRA-SPECIFIC GENETIC VARIATION AMONG *PERKINSUS* SPECIES: IMPLICATIONS FOR SPECIES IDENTIFICATION AND DEVELOPMENT OF MOLECULAR DIAGNOSTICS. **Kimberly S. Reece, Gwynne D. Brown, Karen L. Hudson,** and **Kathleen Apakupakul,** Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, VA 23062, USA.

Perkinsus species are protozoan parasites of marine and estuarine mollusks. Globally, these pathogens have had devastating effects on wild and cultured host populations. There is considerable interest in developing efficient, sensitive, and accurate means of identifying marine pathogens to facilitate monitoring and development of appropriate management practices. The traditional methods for detection of *Perkinsus* species, histological analysis, and the fluid thioglycollate medium (FTM) assay, is time consuming and does not distinguish among recognized *Perkinsus* species. Molecular diagnostic techniques have the potential to improve on the speed and sensitivity of more traditional diagnostic methods. Unfortunately, molecular protocols often prove to be too expensive and technically cumbersome to be practical for routine monitoring purposes. In addition, unless molecular sequence data are available for many strains of a particular species and for closely related species, there is the chance that a molecular diagnostic may give false-negative or false-positive results.

To design genus- and species-specific molecular diagnostics appropriately it is best to obtain DNA sequence information from as many different strains of a particular species and as many species within the genus as possible. We have been examining the DNA sequences for the ribosomal RNA small subunit gene, the internal transcribed spacer (ITS) region, and an anonymous locus from genetic strains of *P. marinus* and from other species of *Perkinsus*. Examination of intra- and interspecific DNA sequence variation, as well as alignments to DNA sequences of other alveolates, has allowed development of *Perkinsus* probes for *in situ* hybridization and primers for use in polymerase chain reaction (PCR).

We have a genus-specific DNA probe that hybridizes to *Perkinsus* species (except *P. qugwadi*) infecting tissues from a variety of hosts. *Perkinsus* cells have been detected in host tissue samples from Asia, New Zealand, Australia, North America, and Europe. PCR primers have been designed based on sequence analysis of the ITS region that specifically amplify *Perkinsus* species DNA from host tissue. In addition, DNA sequencing of the amplified ITS region has allowed identification of *Perkinsus* species. We also have species-specific PCR primers designed from DNA sequence of other areas of the genome to amplify only *P. marinus*. We are testing and optimizing a quick, inexpensive, and sensitive PCR assay for detection of *Perkinsus* species.

HSP70 RESPONSE IN OYSTER *CRASSOSTREA VIRGINICA* EXPOSED TO CD^{2+} AND PAHs SORBED TO ARTIFICIAL SEDIMENTS. **Luis A. Cruz-Rodríguez** and **Fu-Lin E. Chu,** Virginia Institute of Marine Sciences, School of Marine Sciences, College of William and Mary, Gloucester Point, VA 23062, USA. E-mail: lcruz@vims.edu

The induction of Hsp70 has been used as a marker of exposure to pollutants. Ongoing studies are determining if changes in Hsp70 levels could be used as indicators of environmental quality. Our previous studies showed a general increase of total Hsp70 in oysters exposed to field-contaminated sediments containing a mixture of PAHs, heavy metals, and PCBs. To analyze further which contaminants are responsible for the response, we exposed oysters to a heavy metal (Cd^{+2}) or PAHs sorbed to artificial sediments. Oysters were exposed to 15 ppb or 25 ppb Cd^{+2} sorbed to 1 g or 2 g, respectively, artificial sediments, and to 40 µg, 200 µg or 400 µg PAHs sorbed to 1 g, 1.5 g, or 2 g, respectively, artificial sediments daily for 40 days. Concomitantly, to determine the effect of sediments alone, oysters were exposed to 0 g, 1 g, 1.5 g, or 2 g sediments for forty days.

Oysters exposed to 1 g, 1.5 g, or 2 g sediments showed no statistically significant difference in Hsp70 levels compared to the 0 g oysters after forty days (Fig. 1A). Oysters exposed to PAHs-sorbed to the sediments showed a significant increase in the Hsp70 response compared to those exposed to sediments only, but no dose dependency was observed. Oysters exposed to 15 ppb or 25 ppb Cd^{+2} did not show a statistically significant effect as compared to those exposed to sediments only, although the oysters exposed to the highest dose (25 ppb) showed an increase in the Hsp70 levels (Fig. 1B). In a separate study, however, Cd^{+2} sorbed to algae induced a statistically significant Hsp70 response as compared to controls (data not shown). PAHs seem to be more available and/or have a greater exchange between the sediments and the oyster gills than the heavy metal. In summary, based on the above results, Hsp70 potentially could be used as an indicator of exposure to contaminants (PAH and Cd^{+2}) in aquatic environments. This research was funded by the EPA.

COMPARISON OF OYSTER (*CRASSOSTREA VIRGINICA*) CULTURE NURSERY AND GROW-OUT TECHNIQUES. **P. G. Ross, F. X. O'Beirn,** and **M. W. Luckenbach.** E-mail: pg@vims.edu

Along the mid-Atlantic coast, decline of wild eastern oyster (*Crassostrea virginica*) stocks, combined with collateral declines of other fisheries, has prompted development of alternative sources of income from nearshore waters. As a result, interest in oyster aquaculture in the region has increased. Initially, natural reefs were enhanced to take advantage of natural spawning and subsequent settlement and growth of wild oysters. However, the current trend is toward culture of hatchery-spawned oysters. Currently, most commercial oyster aquaculture focuses on three phases: hatchery,

nursery (~3 to 25 mm shell height) and grow-out (~>25 mm shell height-market size). Different techniques are being utilized in each phase. Much research has focused on the hatchery phase with different nursery and grow-out techniques being utilized based on anecdotal comparisons. To further a technical comparison of post-hatchery culture, we evaluated growth and mortality of two nursery and several grow-out techniques. Two nursery techniques were evaluated and compared: (1) fine mesh bags (1/16-in mesh) suspended in the water column via polyvinylchloride (PVC) floats and 1-in × 1-in wire mesh cages, which relies on natural water flow; (2) a forced upweller system (flupsy) utilizing a pump and suspending oysters in the water column via square plastic "silos" with mesh bottoms, which enhances water flow mechanically. Oysters entered both systems at the same size ($\bar{x}$ = 3.08 mm shell height, SD = 0.36) and density (~2,500 oysters). After sixty-three days (April 5 to June 7) oysters had grown to 20.99 mm (SD = 3.02) in the "flupsy" and 18.87 mm (SD = 4.27) in the bag/float system. Low mortality (≤ 1%) was observed for both culture techniques. Although there was a slight growth differential and similar mortality, the most striking difference between these two techniques is their size variation. Variable sizes at the end of the nursery phase can lead to increased handling and sorting, leading to higher costs. In addition, two densities of oysters were compared within the "flupsy" system. Oysters were stocked at ~2,500 oysters and ~120,000 and, after sixty-three days, had grown to 20.99 mm (SD = 3.02) and 18.51 mm (SD = 2.98), respectively. Mortality was similar at both densities (≤ 1%). With similar mortality and size variance, the most important density effect within the "flupsy" system seems to be on growth. Cost comparisons are currently being analyzed for both techniques. Three grow-out techniques are currently being evaluated: (1) mesh bags (3/8-in mesh) in PVC/wire mesh floats (subtidal system); (2) mesh bags in intertidal 1-in × 1-in wire mesh trays with legs that keep oysters ~4 to 6 in off the bottom; and (3) mesh bags placed directly on intertidal shell bottom. Growth, mortality, and costs will be compared between these techniques. It is expected that the advantages conveyed by subtidal growth will be offset by reduced biofouling intertidally.

IMPACT OF WATERFLOW ALTERATION UPON OYSTER GROWTH AND DISTRIBUTION WITHIN ESTUARIES OF SOUTHWEST FLORIDA: IMPLICATIONS FOR MANAGEMENT AND RESTORATION. **Michael Savarese** and **Aswani K. Volety,** Florida Gulf Coast University, 10501 FGCU Boulevard South, Fort Myers, FL 33965, USA. E-mail: msavares@fgcu.edu

Water management practices within the fast growing coastal regions of Southwest Florida have drastically altered natural water quality conditions within estuaries. Two of the more profound changes have been to salinity and nutrient influx. Storm water management has caused some estuaries to receive excessive freshwater; whereas, others receive less because of interruption of sheet flow; and runoff from suburban and agricultural lands delivers higher nutrient loads to some estuaries. Many of these estuaries are targeted for restoration; however, our knowledge of the prealteration conditions is limited. This research employs the eastern oyster, *Crassostrea virginica*, as an indicator species of ecosystem health. The patterns of reef distribution, oyster productivity, and living density compared among pristine and altered estuaries with historically similar hydrologic conditions are used to establish target water quality conditions for restoration efforts. A "spatial homologue" approach is employed, whereby conditions are compared at sites with similar geomorphologies and geographic positions along the estuarine axis.

In the Faka-Union estuary, a system that receives excessive freshwater during the rainy season, the distribution of reefs, the regions of maximum living density, and the foci of maximum oyster productivity are displaced seaward relative to pristine estuaries. Henderson Creek, an estuary receiving greater input of nutrients, has oyster populations with higher mean productivities and higher living densities. Two demographic patterns of oyster productivity are consistently discernable in all estuaries: one of greater variance in biomass, indicating a wider age distribution; and a second skewed heavily toward smaller individuals, indicating greater levels of juvenile mortality. The first pattern persists at sites that experience pulsed release or protracted inundation of freshwater. Faka-Union, because its watershed area has increased significantly because of management practices, exhibits the "freshwater demographic signal" at all but the most downstream spatial homologue. Here, freshwater inundates the estuarine bays for five to six months of the year. The upstream homologues within the estuary below Henderson Creek's weir, whose simplistic design releases pulses of freshwater, exhibit the same freshwater demographic signal. The alternative, "marine demographic pattern" dominates in natural waterflow settings and at the downstream homologues in Henderson. The persistence of small-sized oysters with few older, large individuals, seen in the marine demographic pattern, may be related to the greater occurrence of the disease-causing protozoan *Perkinsus* within environments of higher salinity. Both Henderson Creek and Faka-Union are scheduled for watershed restoration. These patterns of oyster distribution will help establish target environmental conditions.

LABORATORY-BASED TRANSMISSION STUDIES OF QUAHOG PARASITE UNKNOWN (QPX) IN *MERCENARIA MERCENARIA*. **Roxanna Smolowitz, Dale Leavitt, Bruce Lancaster, Ernie Marks, Rhea Hanselmann,** and **Christine Brothers,** Marine Biological Laboratory, 7 MBL St., Woods Hole, MA 02543, USA. E-mail: rsmol@mbl.edu

Disease caused by the protistan QPX has caused severe mortality in aquacultured clams stocks in Massachusetts in the past few years. Before 1995, it had only been identified in Canada, but

had been responsible for mortality in hard clams there. In Massachusetts, up to 44% of clams sampled from a group of severely infected submarket sized clams have shown swellings and nodules in the mantles. Often these lesions are located at the mantle edge, close to, or directly adjacent to the siphon or adductor muscle. Mortality is usually the most severe in the spring and summer months and is associated with the appearance of the gross lesions in the mantle.

The QPX organism has been identified as an unusual member of the phylum Labyrinthulomycota, probably in the family Thraustochytriidae. As QPX proliferates in culture media, it produces abundant mucoid material that binds the individual organisms together. This mucoid material, which is also produced as QPX grows in clam tissues, may prevent phagocytosis and, thus, act as a pathogenic mechanism in the clam tissues. Cultured QPX proliferates best, and produces abundant mucus, at 22 °C.

The method of infection of clams by QPX is unknown. To study the pathogenesis of QPX infection and resultant disease in hard clams, we designed methods for the study of QPX transmission in the laboratory by exposing naive clams to QPX. Methods used involved injecting clams with cultured QPX (with and without the associated mucoid material) in the mantle and pericardial sac, exposing clams to cultured QPX added to the water column and exposing clams to infected clams harvested from severely infected leases. In one set of experiments, clams were placed in a raceway and exposed to filtered seawater at two temperatures and fed two different levels of prepared algae preparations. In the second set of experiments, animals were housed in aquaria at room temperature in the laboratory. Clams used in these experiments were approximately one-year-old.

Interestingly, results to date show that QPX is a directly infective organism. Naive clams, exposed over a twelve-month period to infected clams, developed mild focal QPX infections after three months of exposure, which progressed to severe infections and associated mortality at the end of the experiment. In contrast to the identification of the mantle as the primary location in submarket-sized aquacultured clams, the one-year-old clams first developed infections in the open vascular spaces at the base of the siphon. Clam injected with QPX washed of the mucus coat or exposed to washed QPX in the water column did not develop infections or disease. Studies of clams exposed to mucoid enveloped QPX are ongoing.

FATTY ACID COMPOSITION AND SYNTHESIS OF *PERKINSUS MARINUS* MERONTS AND PREZOOSPORANGIA. Philippe Soudant, Fu-Lin E. Chu, Eric Lund, Jerome La Peyre, and **Aswini Volety,** Laboratoire de Physiologie des Invertebres, Centre IFREMER de Brest BP 70, 29280 Plouzane, France, E-mail: Philippe.Soudan@ifremer.fr

Although parasitic protozoa generally require exogenous sources of essential fatty acids to support their growth and life cycle completion, some are capable of modifying exogenous fatty acids (FAs). Thus, their fatty acid profiles could differ from their culture media and their hosts. To investigate whether *P. marinus* maintains its characteristic fatty acid profile when cultured outside its host, meronts of this parasite were cultivated in two media with different lipids: (1) medium with lipids derived from bovine fetal serum (terrestrial characteristic lipids); and (2) medium with cod liver oil extract (marine characteristic lipids). Results revealed that despite the difference in lipid sources in the two media, FA composition of *P. marinus* meronts cultivated in these two media were similar and were dominated by 14:0, 16:0, 18:0, 18:1(*n*-9), 20:1(*n*-9), 18:2(*n*-6), and 20:4(*n*-6) (Fig. 1) Also, the FA quantity in meronts increased significantly as compared with amounts contained in their media. This suggests that *P. marinus* is able to *de novo* synthesize fatty acids during proliferation. The FA profile of prezoosporangia developed from tissue-associated meronts (PDFTAM) cultivated in fluid thioglycollate medium (FTM), which is deficient in 20:4(*n*-6) and low in 20: 5(*n*-3) (1.4%) and 22:6(*n*-3) (0.6%) resembled its host, although it contained a higher weight percentage of 20:4(*n*-6) (Fig. 2). Surprisingly, the prezoosporangia developed from tissue-free meronts (PDFTFM) cultivated in alternate FTM supplemented with cod liver oil had very high levels of 18:1(*n*-9) and very low levels of 20:5(*n*-3) and 22:6(*n*-3). This research was funded by NSF (MCB9728284).

DEFENSE-RELATED ACTIVITIES IN "NATURAL DERMO RESISTANT" OYSTER STOCKS. Shawn Stickler, Vincent Encomio, Luttrell Tadlock, Jerome LaPeyre, Standish K. Allen, Jr., and **Fu-Lin E. Chu,** Virginia Institute of Marine Science, College of William and Mary, P.O. Box 1346, Gloucester Point, VA 23062, USA. E-mail: stick@vims.edu

The restoration of eastern oyster (*Crassostrea virginica*) populations can be accelerated with the development of strains resistant to dermo disease, caused by the protozoan parasite, *Perkinsus marinus*. To date, this has meant the slow, methodical approach of selectively breeding oysters that have survived repeated exposure to the parasite. The identification of natural "dermo-resistant" wild stocks and related defense activities can benefit selection efforts. Our goals were to identify wild populations with natural dermo resistance or tolerance (NDR) in a common garden experiment and to identify effective defense activities in surviving individuals or populations for use as breeding markers. We collected and spawned oyster from putative resistant and susceptible control stocks from Louisiana (LGT, LHB, LOB) and the Chesapeake Bay (CCR, CRB, CTS), and a hatchery strain (XB). The progeny were deployed in December 1999 at two dermo-enzootic bay sites, Kinsale and Regent for grow-out. Growth and mortality have been

recorded monthly and samples analyzed for dermo prevalence and intensity. As of July 2000, the CRB stock has grown significantly faster at both sites. Mortality levels, unrelated to *Perkinsus* infection, were low in all stocks, and lower in Louisiana (L) than Chesapeake (C) stocks (Fig 1). Infection prevalence and intensity were minimal in all stocks. In July, we collected hemolymph samples to assay hemocyte counts, and we measured plasma protein and lysozyme levels. Protein levels were significantly different between stocks, but lysozyme levels were not (Fig. 2). Future hemolymph samples will also be assayed for *P. marinus* killing by hemocytes, and plasma protease inhibition activity. This research was funded by the NOAA-Virginia Sea Grant-Oyster Disease Research Program.

NATURAL DERMO RESISTANCE AND ITS ROLE IN THE DEVELOPMENT OF HATCHERIES FOR THE GULF OF MEXICO. Shawn M. Stickler, Eric Wagner, Vincent G. Encomio, Standish K. Allen, Jr., and **Jerome F. LaPeyre,** Aquaculture Genetics and Breeding Technology Center (ABC), Virginia Institute of Marine Science (VIMS), P.O. Box 1346, Gloucester Point, VA 23062, USA. E-mail: stick@vims.edu

The value of developing selectively bred dermo-resistant oyster seed for aquaculture and perhaps stock enhancement is obvious. An important step in developing such a value-added product is identifying wild stocks that may have acquired resistance as a result of natural selection. Our goal was to provide clear evidence for the existence of "naturally resistant populations" of eastern oysters *Crassostrea virginica* by using putatively dermo-resistant brood stock from both the Gulf of Mexico and the Chesapeake Bay and determining the inherent resistance of their progeny to dermo disease in a common garden experiment.

We identified and collected putative resistant and susceptible control stocks from both the Gulf (HAB, GTR, OYS) and the Chesapeake Bay (RPP, TSO, COK, XXB) that were spawned at the ABC Gloucester Point hatchery in the summer of 1999. Seed were deployed that fall in Taylor floats at two dermo-enzootic Gulf sites, Grand Isle and Grand Terre. Growth and mortality were recorded monthly, and samples were analyzed for dermo prevalence and intensity using a modified body burden assay.

As of July 2000, dermo prevalence was similar for all stocks; whereas intensity levels in non-Louisiana stock were considerably higher than in Louisiana stocks (Fig. 1). Mortality levels correlated with disease prevalence. Louisiana stocks showed little mortality attributed to disease, whereas, imported stocks experienced high mortality levels. Although there are differences between LA and CB stocks, there is little difference between the two putatively resistant populations from LA and their susceptible control HAB (Fig. 2).

VALIDATION OF DNA-BASED MOLECULAR DIAGNOSTICS FOR THE HARD CLAM PARASITE QPX (QUAHOG PARASITE UNKNOWN) AND THE OYSTER PARASITE SSO (*HAPLOSPORIDIUM COSTALE*). Nancy A. Stokes, Lisa M. Ragone Calvo, Kathleen Apakupakul, Eugene M. Burreson, Inke Sunila, and **Roxanna Smolowitz,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062, USA. E-mail: stokes@vims.edu

Traditionally, the diagnosis of parasitic diseases of bivalve mollusks has primarily relied on standard histological techniques. In recent years, DNA-based molecular diagnostics have been developed for a suite of bivalve parasites; however, for the most part, the utility of these novel tools for shellfish health certification, routine disease monitoring, and basic epizootiological research has yet to be determined and is dependent upon validation with standard diagnostic techniques.

DNA-based molecular diagnostics, DNA probes, and polymerase chain reaction (PCR) assays, were recently developed in our lab for two protistan parasites of bivalve mollusks, QPX (Quahog Parasite Unknown) and *Haplosporidium costale* (Seaside Organism or SSO). QPX is a parasite of hard clams, *Mercenaria mercenaria,* that has been found in cultured hard-clam populations in New Brunswick and Prince Edward Island, Canada and in Massachusetts, New Jersey, and Virginia, U.S.A. The disease has spread south to the United States in the last decade, causing significant clam mortalities in some areas. SSO is a parasite of eastern oysters, *Crassostrea virginica.* The parasite has been reported in oysters on the Atlantic Coast of the United States from Virginia to New Jersey, and organisms histologically similar to SSO have been noted in the northeast up to Maine. Studies using DNA probes recently confirmed the presence of SSO in Connecticut and Massachusetts. Historically, oyster mortalities attributed to SSO were confined to Virginia and Maryland; however, in 1998, the parasite was associated with oyster mortalities in Massachusetts.

The novel molecular diagnostic tools for QPX and SSO have been shown to be sensitive and specific. We are presently conducting studies to validate QPX and SSO PCR assays with the standard histological diagnostic method. Cultured hard clams are being sampled bimonthly from a Virginia coastal embayment and diagnosed for QPX by histological examination and PCR of tissue DNA. Oysters from Virginia, Connecticut, and Massachusetts are being sampled monthly and diagnosed for SSO and *Haplosporidium nelsoni* (MSX) by histological examination and by PCR of hemolymph DNA and tissue DNA. Both studies began in spring 2000 and will continue at least one year. To date diagnostic results between methods have been comparable for QPX; whereas, PCR has resulted in detection of more SSO infections than histological examination.

LOUISIANA'S DERMO ADVISORY PROGRAM: INCIDENCE AND PREVALENCE OF *PERKINSUS MARINUS* ON LOUISIANA'S PUBLIC OYSTER GROUNDS. **John Supan, Ron Dugas, Tom Soniat, Jerome Lapeyre, Ron Thune, John Hawke,** and **Al Camus,** Office of Sea Grant Development, Louisiana State University, Baton Rouge, LA 70803, USA. E-mail: jsupan@lsu.edu

Seed (25 to 75 mm) and market oysters (>75 mm) were collected along coastal Louisiana and analyzed for *Perkinsus marinus* during the summer, 1997 to 1999. The sampling program is funded by the Louisiana Oyster Task Force in response to industry observations of high oyster mortality on the state's public oyster grounds. Sampling locations included Cabbage, Three-Mile Bay, Mozambique Pt., Black Bay, Telegraph Pt., Bay Crabe, Bay Gardene, Lonesome Island, Hackberry Bay, Sister Lake, Bay Junope, Vermillion Bay, and Calcasieu Lake oyster reefs. The assay utilized rectal tissue in Ray's Fluid Thioglycollate Media, using the Mackin scale for qualification. The sampling program is the basis for the state's Dermo Advisory, printed annually in the task force's newsletter to assist industry in the management of private oyster leases. State fisheries managers also use the data to manage the public oyster grounds.

Perkinsus intensity varied annually at each site and oyster category and was greater during 1997 than subsequent years. On the prime grounds in the eastern portion of the coast, seed oysters ranged from 0.1–1.9 weighted incidence, with eight out of nine stations >1.0; prevalence ranged from 16–100%, with six stations >90%. Market oysters ranged from 0.6–2.0 and 59–100%, respectively. Vermillion Bay was the lowest site during all three years. Incidence and prevalence has declined during 1998 to 1999 at most stations; whereas, Hackberry Bay, in the central portion of the coast, had the highest infection levels for market oysters during 1999. High market oyster mortality and heavy spatfall have been prevalent during these latter years.

Sampling for 2000 is underway and will be incorporated in the annual Dermo Advisory. Preliminary stock assessments indicate greater mortality in market oysters a most stations. Higher levels of *Perkinsus* are expected because of severe drought. The advisory will soon utilize the new DermoWatch program now online, funded by the Gulf Oyster Industry Program.

DISEASE STATUS AND PHYSIOLOGICAL RESPONSES OF OYSTERS AS INDICATORS OF WATERSHED ALTERATION EFFECTS IN SOUTHWEST FLORIDA ESTUARIES. **Aswani K. Volety, Michael Savarese,** and **S. Gregory Tolley,** Florida Gulf Coast University, 10501 FGCU Boulevard South, Fort Myers, FL 33965, USA. E-mail: avolety@fgcu.edu

Southwest Florida possesses one of the country's fastest growing populations. Consequently, watersheds are heavily managed to accommodate development. Studies on the effects of watershed alterations involving valued ecosystem components, such as oysters, are lacking, but clearly necessary. Using the oyster, *Crassostrea virginica*, as an indicator species, we are investigating ecosystem-wide health effects of watershed management practices in altered (Faka-Union, Henderson Creek, and Caloosahatchee River) and pristine (Blackwater River) estuaries. Currently, watershed management involves the simple opening and closing of weirs in the estuaries, thus delivering pulses of freshwater during rainy season and decreasing freshwater input into the estuaries during the dry season. This results in the estuaries being mostly freshwater when the weirs are open and mostly higher salinity water when the weirs are closed. Measurements of disease prevalence of *Perkinsus marinus*, condition index, and substrate suitability of healthy oyster reefs as essential fish habitat are underway using a "spatial homolog approach" (comparing conditions among hydrologically and geomorphically similar points between estuaries) along salinity gradients. Preliminary results indicate that in summer months, mean prevalence of *P. marinus* infection in oysters varied between 33–73% depending on the location; mean condition index varied between 2.4 to 4.7 during July 2000, although lower disease prevalence was noted in oysters from homologs 2 and 3 in Faka-Union and is likely attributable to the greater freshwater input into the estuary, homolog 1 (upriver) was devoid of oysters because of freshwater-induced mortality. The lower disease prevalence in homologs 3–5 (down river) in Blackwater River and Henderson Creek may be attributable to the disease-induced mortality of these heavily infected oysters during the summer months. This project represents the first study of watershed alteration on oysters in Southwest Florida and will help provide target environmental conditions for restoration efforts.

PARTIAL PURIFICATION AND CHARACTERIZATION OF LYSOZYME-LIKE PROTEINS FROM THE PLASMA OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*. **Aswani K. Volety, Fu-Lin E. Chu,** and **Luis Cruz-Rodríguez,** Florida Gulf Coast University, College of Arts and Science, 10501 FGCU Boulevard South, Ft. Myers, FL 33912, USA.

Lysozyme(s) (and lysozyme-like proteins) are shown to be involved in a broad battery of such defense mechanisms as bacteriolysis and opsonization in both vertebrates and invertebrates. The action of the enzyme on microorganisms is mediated by the hydrolysis of β-(1-4)-glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in bacterial cell walls. Our previous work showed that lysozyme activity in oysters, although positively correlated with low temperatures and salinities, was negatively correlated with *Perkinsus marinus* infections in oysters. Conditions of temperature below 10°C and salinity below 10‰ are not favorable to the parasite. This suggests that lysozyme may be involved in imparting a protective role in oysters against *P. marinus* infections. As a first step in elucidating the role of lysozyme in oysters' defense, we have partially purified lysozyme-like enzyme(s) from the plasma of oysters, *Crassostrea virginica*.

Lysozyme-like proteins were purified by cation-exchange chromatography using carboxymethyl cellulose, followed by concentration, desalting, and separation of proteins using Centricon 3 and Centricon 30 filtration. The proteins were eluted using a step gradient acetate buffer (0.01 M) with 0–0.8 M NaCl. Lysozyme activity was measured spectrophotometrically at 450 nm by the lysis of *Micrococcus lysodeikticus* suspension in phosphate buffer. Preliminary analysis using SDS-PAGE revealed the presence of two lyzozyme-like proteins with apparent molecular weight of 18.2 KDa and 38.8 Kda, respectively (Fig. 1). The separated proteins based on molecular weight cut-offs show the ability to lyse the bacterium *Micrococcus lysodeikticus*. We hypothesize the 38 KDa protein to be a dimer of the 19 KDa protein. Currently, we are investigating the effects of the separated proteins on *P. marinus* and various bacterial species. This research was funded by the NOAA-Virginia Sea Grant-Oyster Disease Research Program.

SUSCEPTIBILITY OF CULTURED *PERKINSUS MARINUS* AND *VIBRIO PARAHAEMOLYTICUS* CELLS TO HEMOCYTES OF EASTERN OYSTER, *CRASSOSTREA VIRGINICA*. **Aswani K. Volety,** Florida Gulf Coast University, College of Arts and Sciences, 10501 FGCU Boulevard South, Fort Myers, FL 33965, USA.

Oysters possess a very effective defense system comprising cellular and humoral defenses. Various measurements of the ability of oysters to generate a defense response against pathogenic and nonself particles have been developed. However, the relationship of these measurements to the actual defense of the organisms is unclear. Therefore, a colorimetric assay was developed to assess the ability of oyster hemocytes to kill *Vibrio parahaemolyticus, in vitro*. This assay was subsequently modified to investigate the ability of oyster hemocytes to the kill cultured *Perkinsus marinus* isolated from various geographical locations.

Hemocytes from Rhode Island and Florida oysters were used to assess their ability to kill *P. marinus* isolates from Connecticut, Delaware, Maryland, Virginia, Louisiana, and Texas. In addition, seasonal influences on the ability of hemocytes to kill *P. marinus* and *Vibrio parahaemolyticus* was examined by monthly sampling of Florida oysters. Hemocytes from both stocks of oysters were able to reduce viability of *P. marinus* cells by 25–90% depending on the isolate. *P. marinus* isolate from Virginia was the most susceptible; whereas, isolates from Louisiana, New Jersey, and Connecticut were less susceptible to hemocyte killing. Variation in the results seemed to result from differences in the susceptibility of the isolates rather than the ability of hemocytes from two oyster stocks. Killing of *P. marinus* by oyster hemocytes was lowest during peak summer months (July–August) and increased through the winter months. This trend contrasted with low winter and high summer bactericidal activity of hemocytes against *Vibrio parahaemolyticus*. The differences in the susceptibility may indicate different killing mechanisms for bacteria and *P. marinus*.

The assays developed for assessing killing of bacteria and *P. marinus* are relatively simple, inexpensive, reproducible, and enable numerous replications owing to the low numbers of hemocytes required to carry out the assays. These techniques could be used in investigating the role of environmental factors, mechanisms of action, and contaminant stress on host–parasite interactions.

EFFECT OF PERKINSOSIS ON THE ENERGETIC PHYSIOLOGY OF THE CLAM *RUDITAPES DECUSSATUS*. **Antonio Villalba** and **Sandra M. Casas,** Centro de Investigacións Mariñas, Xunta de Galicia, Aptdo. 13, Vilanova de Arousa 36620, Spain. E-mail: villalba@cimacoron.org

Perkinsosis has been considered a threat for Galician clam industry since its detection in late 1980s. A program is being developed to evaluate the potential effect of the disease in clam *Ruditapes decussatus* populations of the Galician coast. One of the approaches is estimating the effects of parasitization by *Perkinsus atlanticus* on clam energetic physiology. In a first experiment, fifty clams (40 to 50 mm in length) were taken from a natural bed (Ría de Arousa, Galicia, NW Spain) with high prevalence of this parasite. Clams were allowed to acclimatize for two weeks to laboratory conditions (15°C and 35 ppt) and fed with cultured phytoplankton. Temperature value of 15°C was chosen as a midvalue of the range in Galician Rías (10 to 20°C). Respiration and clearance rates of each clam were estimated. Then, each clam was processed for disease diagnosis. The intensity of infection by *Perkinsus atlanticus* was estimated both through histological techniques and incubation of a gill lamella in fluid thioglycollate medium. Both physiological rates showed a decreasing tendency as infection intensity increased. Nevertheless, differences between infection intensity categories were not statistically significant, with either diagnostic method.

In a second experiment, sixty clams (42 to 47 mm long) from the same bed were allowed to acclimatize for six days to laboratory conditions. Water (15°C and 33 ppt) was continuously pumped from the Ría de Arousa into the acclimatization tanks. In this occasion, respiration, clearance and excretion rates, and absorption efficiency of each clam were estimated. The total *Perkinsus atlanticus* body burden was calculated in addition to the two previous diagnostic methods. No significant effect of either infection intensity or *P. atlanticus* body burden on any of the physiological parameters was found. Those results support that perkinsosis does not broadly affect clam scope for growth, at least at 15°C. Nevertheless, in a previous study, a statistically significant decrease (up to 25%) of clam condition index was detected only when infection intensity was heavy.

Concurrent results allow rejecting that perkinsosis is responsible for clam mortality in the study bed, which is one of the most heavily affected by *P. atlanticus* in Galicia. Clam mortalities associated with perkinsosis have been described in warmer European

areas. New experiments will be performed at 20°C in September 2000, when seawater temperature in the clam bed is around that value to test if seawater temperature modulates the effect of perkinsosis on host energetic physiology.

GROWTH, GONAD DEVELOPMENT, AND MORTALITY OF GAMMA-IRRADIATED JUVENILE EASTERN OYSTERS. **Eric Wagner, Jerome La Peyre, John Buchanan, John Supan,** and **Terrence Tiersch,** Department of Veterinary Science, Louisiana State University, 111 Dalrymple Building, Baton Rouge, LA 70803, USA. E-mail: eric.wagner@mindspring.com

Gamma irradiation has been shown to cause sterility and to increase growth in a variety of organisms, possibly because of partial or complete blockage of gametogenesis and reallocation of energy to somatic tissues. Sterilization of oysters would be advantageous, because meat quality and yield would be improved during spawning season. Moreover, an increase in growth rate may allow oysters to reach market size before succumbing to diseases. The development of a sterilization method will also be essential for use of transgenic oysters. The objective of this study was, therefore, to determine the effect of gamma irradiation on the growth, gonadal development, and mortality of juvenile oysters.

Juvenile oysters were exposed to 5 and 10 krad of cobalt-60 gamma rays using a Sheperd irradiator at a rate of 1353 rad/min. Three groups of four hundred oysters were irradiated separately at each dose. Control oysters were handled similarly to the irradiated oysters, except they were not exposed to gamma rays. Each group of oysters were placed in Taylor floats (2-in × 3-in) and grown for one year at Grand Isle, Louisiana. The number of dead oysters in each of the nine floats and the size of fifty oysters per float were determined monthly. The gonadal development of irradiated and control oysters (fifteen per dose) was also evaluated every month from March to June.

The oysters receiving 10-krad of radiation experienced significantly greater mortality than either the 5-krad or control oysters during nine months of grow-out. Significant differences in growth occurred between the control oysters (63 ± 1 mm), 5-krad oysters (54 ± 5 mm) and the 10-krad oysters (42 ± 1 mm). Significant differences in mean gametic stage among the three treatments were detected for the oysters sampled in April ($P = 0.0066$) and May ($P = 0.0032$). In each month, oysters irradiated at 10-krad had significantly lower gametic stages than did control oysters ($P < 0.05$). No differences among treatments were detected for oysters sampled in June ($P = 0.6940$). Irradiation affected sex ratio among treatments as well. Control oysters were 68% female and 32% male; 5-krad oysters were 45% female, 45% male, 2% hermaphrodite, and 6% undifferentiated; 10-krad oysters were 20% female, 60% male, 2% hermaphrodite, and 18% undifferentiated. These data indicate no production advantage to irradiation of juvenile oysters at 5 or 10 krad, but provides insight into possible effects on sex ratio and gonadal development.

EXPERIMENTS IN DETERMINING OPTIMUM SIZE FOR PLANTING HATCHERY PRODUCED OYSTER *CRASSOSTREA VIRGINICA* SEED. **Richard K. Wallace, David B. Rouse, F. Scott Rikard, Jeffrey C. Howe, Blan A. Page, Donald B. Gruber,** and **John K. Dunne,** Auburn University Marine Extension & Research Center, 4170 Commanders Dr., Mobile, AL 36615, USA. E-mail: rwallace@acesag.auburn.edu

Hatchery-produced oyster seed has been available in the Gulf of Mexico region for several years. However, the oyster industry has been reluctant to invest in seed without more information on the relationship between cost of seed and survival. We investigated the survival of different size (≤5 mm, 6–10 mm, 11–15 mm, and 16–20 mm) remote set (on, shell) and cultchless oyster seed planted in replicated units at two sites (low salinity: 10–30 ppt and high salinity: 19–29 ppt) in Mobile Bay, Alabama. Water quality (temperature, salinity, and oxygen) was monitored continuously at the sites, and laboratory experiments were performed to examine survival under anoxic conditions.

Mean survival after thirty-two weeks for remote set seed at the low salinity site ranged from 43% for 11–15 mm seed to 51% for 16–20 mm seed (Fig. 1). There was no significant difference in survival among the four size groups. Cultchless seed at both sites and remote set seed planted at the high salinity site did not survive the first two-week sampling period. Predation seemed to be the cause of mortality.

Mean height of seed after thirty-two weeks varied from 48 mm (range = 23–63 mm) for the ≤5 mm group to 55 mm (range = 37–75 mm) for the 16–20 mm group. There was no significant difference in height among the three larger seed sizes, but the ≤5 mm group was significantly smaller than the other three seed sizes. Very low dissolved oxygen (<1 ppm) occurred at the low salinity site with varying durations (0.5–19.5 h). Preliminary laboratory studies indicated that the LT-50s for anoxic conditions (at 30°C and 15 ppt) ranged from 94 to 98 h, and there was no relationship between the LT-50s and size of seed.

EVALUATION OF A MARSHLAND UPWELLING SYSTEM FOR THE TREATMENT OF RAW DOMESTIC WASTEWATER FROM COASTAL DWELLINGS. **Robert E. Watson, Jr., Kelly A. Rusch,** and **Tingzong Guo,** Louisiana State University, Department of Civil and Environmental Engineering, Engineering Lab Annex Building, Baton Rouge, LA 70808, USA. E-mail: rwatson@unix1.sncc.lsu.edu

The oyster industry depends on a healthy water environment. Waters with fecal coliform bacteria concentrations in excess of the National Shellfish Sanitation Program standard of 14 MPN $^{FC}/_{100\ mL}$ are closed to harvesting. Improperly treated sewage from coastal dwellings is a major source of bacterial contamination. Conventional wastewater treatment strategies are not viable options for many of these dwellings because of their remote locations, sporadic usage, and proximity to water tables that are typi-

TABLE 1.

Fecal coliform removal under employed injection rates (Q = 0.25, 0.5, 1.0 gpm).

Flowrate	Influent fecal coliform concentration	Effluent fecal coliform concentration	Required vector feet of travel	Exceedance probability (≥ 14 $^{FC}/_{100\ mL}$)
0.25 gpm	326,700 $^{FC}/_{100\ mL}$	2.14 $^{FC}/_{100\ mL}$	8.8 ft	11%
0.5 gpm	930,400 $^{FC}/_{100\ mL}$	3.08 $^{FC}/_{100\ mL}$	10.3 ft	5%
1.0 gpm	559,200 $^{FC}/_{100\ mL}$	7.25 $^{FC}/_{100\ mL}$	11.7 ft	23%

cally at or above the ground surface. Thus increased efforts are being focused upon developing innovative methods to treat domestic wastewater effectively in poorly sewered coastal areas. The primary objective of this research was to evaluate the efficacy of a marshland upwelling system in removing fecal pathogens from raw domestic wastewater.

Wastewater was injected intermittently down a 15-foot deep well under three flowrates. Density gradients created between the wastewater and relatively dense native saline groundwater confined the injectant within a limited area and forced it vertically toward the ground surface, allowing the natural soil matrix to function as an upflow sand filter removing fecal pathogens. Concentrations in the 5-ft monitoring wells were used as a conservative estimate of effluent counts. Fecal coliform removal was assumed to follow first-order decay, allowing the required travel distance for influent concentrations to fall below the 14 MPN standard to be calculated. Best-fit probability density functions were determined for effluent fecal coliform concentrations under each flowrate and used to predict the probability of effluent counts exceeding the 14 MPN standard (see Table 1).

Two types of failure are believed to exist: (1) isolated failure created by accelerated intermittent injection flowrates, that may cause localized pressures to build and create transient episodes of channelization; and (2) catastrophic failure induced by excessive hydraulic loading rates that cause global increases in fecal concentrations because of reduced hydraulic retention times and the corresponding widespread declines in pathogen removal. Although treatment efficacy did vary with injection flowrate, and isolated instances of abnormally high fecal counts were observed under the 1.0 gpm flowrate, the MUS never experienced a catastrophic failure during the thirteen-month evaluation period.

MHACS: MARINE HABITAT ACOUSTIC CHARACTERIZATION SYSTEM. A PROGRAM FOR THE ACQUISITION AND INTERPRETATION OF DIGITAL ACOUSTICS TO CHARACTERIZE OYSTER HABITAT. Charles A. Wilson and **Harry H. Roberts,** Coastal Fisheries Institute, Department of Oceanography and Coastal Sciences, CCEER, Louisiana State University, Baton Rouge, LA 70803, USA.

Coastal Louisiana, like many deltaic land masses, faces continued landscape alteration from natural processes and anthropogenic impacts that affect estuarine habitat. Steps are being taken at both state and federal levels to slow/mitigate these changes. Most promising of these strategies is river diversions that introduce freshwater and sediment to river-flanking environments (lakes, bays, and associated marshlands). Two such diversion projects (Caenarvon and Davis Pond), planned by Louisiana and the U.S. Army Corps of Engineers, are designed to nourish marshes with water and sediment as well as to help establish ideal salinities over historic oyster grounds. Critical to the success of these programs is a rapid and accurate means to qualify and quantify changes in oyster habitat.

Digital high-resolution acoustic instrumentation linked to state-of-the-art data acquisition, and processing software was used to build a baseline of information for evaluating future changes in shallow and shelf water bottoms with special emphasis on oyster habitats. Application of digital side-scan sonar (100 and 500 kHz), a broad-spectrum sub-bottom profiler (4–24 kHz) for rapidly acquiring water column, surficial and shallow subsurface data has now been accomplished. In our most recent study, geo-referenced side-scan sonar mosaics of a Louisiana estuary were incorporated into a GIS data base. These datasets "calibrated" with surface sampling, coring, and other "ground truthing" have established that numerically indexed acoustic reflectance intensities correlate closely with surface shell and oyster reef density. With image-processing techniques to analyze mosaic reflectance patterns, we estimated the percentage and total acreage of several bottom types. Results were calibrated with field collected ground truth measurements.

PRODUCTION AND EVALUATION OF MEIOSIS I AND II TRIPLOIDS IN THE HARD CLAM, *MERCENARIA MERCENARIA*. Huiping Yang, Jian Wang, and **Ximing Guo,** Haskin Shellfish Research Laboratory, Institute of Marine Coastal Science, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349, USA. E-mail: hyang@hsrl.rutgers.edu

Triploid mollusks grow significantly faster than normal diploids in most species studied so far. Results from clams are mixed. Triploid dwarf-surf clams grow 72% faster than normal diploids; whereas, triploid soft-shell clams had the same or slower growth rate than diploids. In the hard clam, *Mercenaria mercenaria*, previous studies show that triploids grow faster than diploids at four years of age, but not during the first two years. Triploids may perform differently under different environments, and triploid hard

clams have not been evaluated in New Jersey. Different types of triploids, such as meiosis I triploids, meiosis II triploids, and mated triploids, also differ in growth performance. The objective of this study is to produce two types of triploids by inhibiting polar body 1 (PB1) and polar body 2 (PB2), respectively, and evaluate their growth performance in New Jersey.

Three experimental groups were produced: a diploid control, a PB1 triploid group, and a PB2 triploid group. Cytochalasin B (CB, 1.0 mg/L) was used as the induction agent. Four replicates were produced using different sets of brood stock. Ploidy was determined by counting chromosome number at two- to four-cell stage and by flow cytometry (FCM) at D-stage and beyond. High levels of triploids were produced with both methods, ranging from 62 to 83% in PB1 inhibition groups and from 82 to 100% in PB2 inhibition groups. Tetraploids and aneuploids were also produced in PB1 groups, but became undetectable at late larval stages by FCM. Survival of larvae in PB1 groups was lower than that in PB2 groups. Experimental clams will be deployed at two sites in New Jersey and sampled at regular intervals for ploidy determination and growth measurements. Data from the first two sampling dates, three and six months of age, are presented.

A STUDY OF THE EFFECT OF DERMO DISEASE *PERKINSUS MARINUS* ON EASTERN OYSTERS *CRASSOSTREA VIRGINICA* IN THE PATUXENT RIVER, MARYLAND WITH THE HELP OF COMMUNITY VOLUNTEERS. George R. Abbe, Brian W. Albright, Erin S. Woodrow, and **Shannon B. Campbell,** Academy of Natural Sciences, Estuarine Research Center, 10545 Mackall Road, St. Leonard, Maryland 20685, USA. E-mail: abbe@acnatsci.org

In studies of natural oyster populations affected by dermo disease in the Patuxent River Maryland, we have examined live oysters and boxes from dredged samples over time to gain some understanding of mortalities associated with disease. Not knowing the accuracy of the box- to-live ratio, we suspended three trays of one hundred oysters each from piers along a 33-km salinity gradient in the Patuxent in January 1997 and measured growth and survival monthly during the year. After a year, it was evident that oyster mortalities were highly correlated with salinity and dermo infection.

We thought that by increasing the number of sites along this salinity gradient we could gain a better understanding of oyster–salinity–disease interactions but lacked the resources and time to do so. With the help of community volunteers who donated piers and/or time and effort, we were able to conduct a similar study during 1998 at twenty sites along the Patuxent River. Each tray was stocked with one hundred oysters from the nearest natural oyster bed. By January 1999, it was evident that oyster mortalities decreased in an upriver direction with decreasing salinity as did the prevalence and intensity of dermo as measured by an infection index. Dermo levels in tray-held oysters were highly correlated with disease levels on nearby natural bars, and mortalities in trays were also highly correlated with estimated mortalities from dredged samples based on box-to-live oyster ratios. One of the most valuable results of this study was the increased awareness of the participants of environmental issues in their own communities. They learned to collect data year round at the same time each month and how their efforts fit into the larger riverwide picture.

Relationship of dermo infection index to oyster mortalities in trays as compared to those salinity in the Patuxent in September 1998, estimated from natural beds during 1998.

IMPROVING ACCURACY IN THE DETERMINATION OF MEAT CONDITION INDEX FOR THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*. Brian W. Albright and **George R. Abbe,** Academy of Natural Sciences, Estuarine Research Center, 10545 Mackall Road St., Leonard, MD 20685, USA. E-mail: Albright@acnatsci.org

The meat condition index (MCI) of a bivalve is a numerical representation of the quality of its soft tissue. Based on the percentage of the internal shell volume occupied by an oyster's soft body tissue, enumeration of a quantitative index is possible. Older methods sought to measure shell cavity volume volumetrically; however, this is not practical. In 1982, Lawrence and Scott developed a method to determine MCI gravimetrically, where shell cavity capacity is determined by the difference between whole oyster weight and empty shell weight after drying for 24 h.

After using this method for more than fifteen years, we questioned its accuracy. Because any water contained in the shell itself (not between the valves) was included in the whole oyster weight, it seemed this should be included in the weight of the empty shells as well. Drying shells for 24 h could make the shell cavity appear larger than it really was, resulting in a reduced meat condition.

To determine the significance of weighing shells at 0 h verses 24 h of drying, meat condition indices were first determined based on true cavity volume using a moldable material of known density and then compared to the two methods. Weighing shells immediately after processing was determined to more accurately estimate cavity capacity whenever shells lost more than 3% of their weight because of drying.

Because oyster shells are highly variable with respect to porosity, we set out to determine the average weight loss of the valves after 24-h drying time. Over a 3-y period, monthly collections from many oyster bars in the Patuxent River resulted in the examination of 1,749 oysters, of which 74.3% lost more than 3% shell weight. Several other sites in the Chesapeake Bay were also examined yielding similar results.

Weighing shells at 0 h increased accuracy for most of the oysters we examined and also saved time and space, because shells do not need to be held for an additional 24 h for weighing. To date, this method has neither been tried for other species nor over the entire range of *C. virginica*. Differences in shell morphology and fouling communities may influence shell porosity, favoring one technique over the other.

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COVER PHOTO: *Cenchritis muricatus* among leaves of the coastal shrub *Sesuvium portulacastrum;* photographed at Swan Island in the Caribbean, March, 1973. This snail is one of several species of tropical Caribbean littorinids and occupies the highest position on rocky intertidal shores. (Joseph C. Britton)

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4th International Symposium on Abalone Biology, Fisheries, and Culture

University of Cape Town
Cape Town, South Africa

February 6–11, 2000

Guest Editor:

Peter A. Cook
Zoology Department
University of Cape Town
Rondebosch, South Africa

PREFACE

The 4th International Symposium on Abalone Biology, Fisheries, and Culture was held at the University of Cape Town, South Africa from 6th to 11th February, 2000. The conference attracted about 250 delegates from at least 15 different countries, and was successful in bringing together scientists, entrepreneurs, divers, aquaculturists, and government representatives. This Special Issue of the *Journal of Shellfish Research* contains many of the contributions presented at the conference.

The Cape Town Abalone Symposium was the fourth in a series that started in La Paz, Mexico in 1989. The second was held in Hobart, Tasmania in 1994 and the third in Monterey, California in 1997. It was decided, at the Monterey symposium, that a three-year interval between symposia was appropriate in view of the rapid changes that were occurring worldwide in abalone fisheries and in farming techniques. The large quantity of new information presented at the Cape Town conference proved this decision to be correct.

The choice of Cape Town as the venue for the 4th International Abalone symposium was appropriate due to the fact that the South African abalone fishery is, perhaps, one of the most over-exploited in the world, principally because of illegal exploitation by poachers. The occasion of the symposium was used to highlight this problem, and extensive media coverage provided a platform for scientists to sound alarm bells about the state of the fishery. In addition, the south-western region of the country has developed as the center of South Africa's abalone farming industry and conference delegates were able to benefit from visits to local farming enterprises.

The symposium was opened with an interesting and thought-provoking review of the international supply, markets, and pricing of abalone products. The rest of the symposium was divided into a number of sections including abalone aquaculture, nutrition, genetics, diseases, stock assessment, and fisheries management. This volume contains a selection of the papers from each section and all papers have been through the usual stringent refereeing and review process applied by this journal. I wish to thank the authors who submitted manuscripts for publication in this volume and, in particular, I also wish to thank the numerous referees who contributed their time to review the papers.

The 5th International Abalone Symposium will be held in China in 2003.

Peter Cook
Editor

DEDICATION

This volume is dedicated to the memory of Mia Tegner, whose work on abalone contributed enormously to our understanding of the biology of this unique creature.

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WORLD ABALONE SUPPLY, MARKETS AND PRICING: HISTORICAL, CURRENT AND FUTURE

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ABSTRACT The world supplies of wild-caught and cultured abalone are considered with implications of the past, present and future. Much of the data available in recent years, from various government sources, and even FAO, have often been inadvertently misleading. Reporting has often combined a number of dissimilar abalone products (fresh in shell, frozen meat, canned, dried, etc) resulting in misleading results. Surprising numbers result when an effort is made to standardize production and export information for both the commercial catch and cultured product. Factors affecting abalone FOB and CNF prices are discussed. Different market forms (live, fresh, frozen, canned, dried) affect pricing, and price is also influenced by processing and packaging as well as economic conditions. Suggestions are made for value added abalone products and the challenges of sustaining world demand at premium prices are discussed.

KEY WORDS: abalone, abalone market, abalone prices, abalone process and packaging, abalone future

INTRODUCTION

World abalone supply statistics have not all been accurate over the past 30 years. In a reexamination of the statistics the industry has used for many years, a need for some substantial adjustments in both the fisheries and the cultured sectors has been found. Annual data for some countries has substantial gaps in reporting. Whilst some countries have reported tonnage as "in shell", others report "meat only". This distinction alone can distort the comparative total production of an individual country by two to three hundred percent. To complicate the analysis further, export and import data often combine a number of dissimilar abalone products (fresh in shell, frozen meat, canned, dried etc) into a single number for tonnage and value. In an attempt to standardize the reporting of the world abalone supply, the following definitions are applied:

Abalone fisheries: The total allowable annual commercial landing quota (country by country) expressed in terms of "in shell" weight. This category would include the planting of seeds in large areas of the sea (e.g. in Japan) wherein the sea bottom has not been prepared with man-placed rocks or structures. This definition does not include the legal sport catch or any illegal catch worldwide.

Cultured abalone: (expressed in terms of "in shell" weight) includes both the farming of abalone on land or in the sea—contained in man-made tanks, nets or structures (Intensive culture) and sea planting of abalone seeds in artificially arranged substrate or structures, with or without added food (Extensive culture).

The illegal catch: Any harvest of abalone beyond the total allowable annual landing quota. To discuss the topic of world supply without consideration of the illegal catch would be grossly misleading (Murphy 1999).

TEN-YEAR FISHERIES COMPARISON

In order to compare the abalone fisheries and cultured totals on an "apples with apples" basis, we must apply a standard "in shell" weight. Retroactive reporting (in particular China, but other countries as well) dramatically changes the cultured abalone totals (New 1999). We now know that the FAO Yearbook of Fisheries Statistics had been reporting China's production of several groups of cultured mollusks, including abalone, as shelled or shucked weight, instead of their "in shell" live weight equivalent, which should be normal practice in submitting statistics to FAO. This has grossly underestimated both cultured abalone production and abalone fisheries. Adjustments must, therefore, be made to previous reporting over this 10-year timeframe.

Figure 1a shows that the worldwide catch from abalone fisheries has declined by about 30% over the past 10 years. In 1989 worldwide abalone catch was reported as 12,995 mt but, based on the assumptions given earlier concerning incorrect reporting, this has been adjusted to 14,830 mt. It is estimated that the catch in 1999 was 10,150 mt.

From Figure 1b it can be seen that Australia is the only country where abalone fisheries have increased over the past 10 years. Significant declines have occurred in countries such as Mexico, the U.S.A., and Japan.

TEN-YEAR COMPARISON OF CULTURED ABALONE

The situation with regard to cultured abalone is completely different (Figures 2a and 2b). Over the ten year period from 1989 to 1999, whilst abalone fisheries declined by about 30%, the production of cultured abalone increased by over 600%. In 1989 cultured abalone production was reported as 689 mt (adjusted to 1,220 mt), whilst in 1999, it is estimated that production was 7,775 mt. Of the total estimated 1998 cultured abalone world production of 7,165 mt, Asia accounted for 5,500 mt or 75% of that total. Within Asia, the vast majority of the cultured abalone production is from China and Taiwan.

ABALONE PRICING

With regard to the price of abalone products, it is interesting that the F.O.B. and C.N.F. prices for similar species and sizes tend to equate worldwide. Abalone is marketed in many different forms, each of which is priced differently. Figure 3 illustrates, diagrammatically, a range of prices that are quoted for different forms of abalone, but the examples given below will show that, when processing and marketing costs are taken into account, these prices tend to revert back to a similar "in shell" price of about US$32/kilo.

Example one: cultured Red abalone in the 90-mm-size range. (The principle is the same with other species when adjusted for the size/weight ratio of that species.) Many of the Asian traditional recipes call for dried abalone as their preparations began thousands

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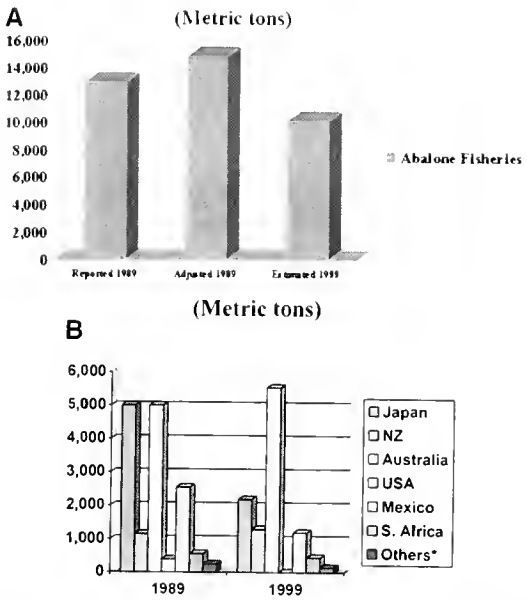


Figure 1. (A) Worldwide abalone fisheries: ten-year comparison; (B) Abalone fisheries country by country: ten-year comparison.

of years before refrigeration. There are a few special (often secret) processing methods creating a dried product which sells for a minimum of US$700 per kilo and as much as $12,000 per kilo or more. This dried abalone is not to be confused with the "standard" sun dried product that sells at a lower price. The preparation of this specialty-dried abalone is almost ceremonial, often prepared with unique coals and slow cooking in ceramic cooking utensils. Only about 10% of the "in shell" weight remains in the dried product. The preparation process is highly labor-intensive and takes months to complete. If the processor pays US$32/kilo "in shell", and sells the premium product for US$700/kilo, he is making only a reasonable profit for the effort.

Example two: a live 90-mm abalone at the Japanese wholesale

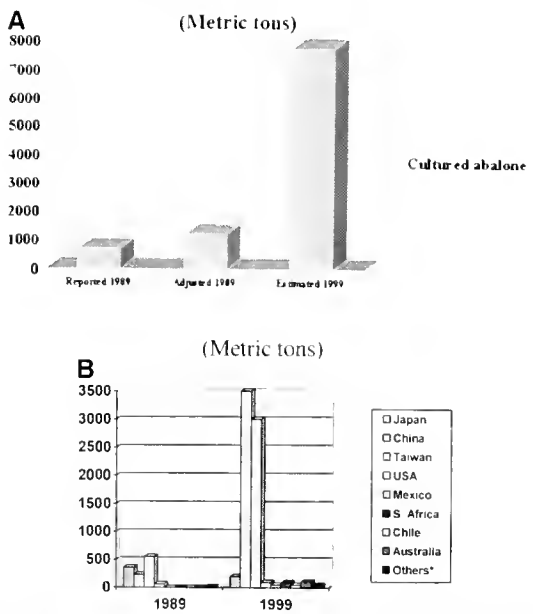


Figure 2. (a) Worldwide production of cultured abalone: ten-year comparison; (b) Cultured abalone production country by country: ten-year comparison.

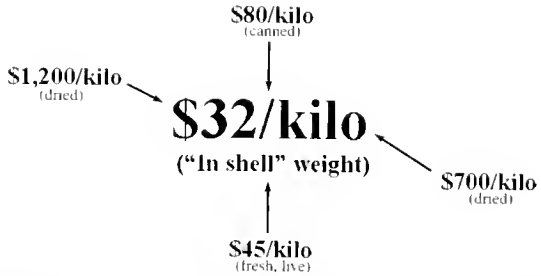


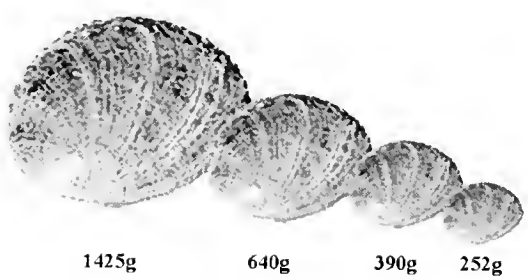
Figure 3. Prices for different market forms of abalone (for explanation see text).

(auction) markets. There are fisheries pricing services that regularly publish an average of 10 major Japanese wholesale markets (Sunee 1999). For the sake of comparison, we will use a price of 4,700 yen/kilo. This equates to approximately US $45/kilo. Does that mean you might sell to these markets at a C&F level approaching these prices? Not by a long shot: there are auction commissions, wholesaler commissions, trading company commissions, local transportation, customs and duty—all to be deducted from your selling price along with possible mortality deductions. In effect, $45/kilo equates closer to $32/kilo.

Example three: weight losses during canning (illustrated in Figure 4). If the "in shell" weight is 1425 g (*H. rufescens*, 8 pcs, 100 mm "in shell", for example) shucking will yield about 640 g of meat which, after cleaning and trimming, will yield approximately 390 g. After cooking the meat, you will find only 252 grams. In effect, after shrinkage and canning cost, a $20 tin of 252 g meat equates to an "in shell" price of as low as only $12/kilo. Without the continued development of premium product and brand name tins, canned abalone will remain as a commodity with relatively lower prices.

ALTERNATIVES TO WORLD COMMODITY PRICING

As illustrated in Figure 5, the world abalone market is dominated by Asian countries. Prices are currently driven by a handful of Asian nations, guided by their historical and changing customs, preparations, populations and economies. An important additional influence is that of Asian populations living elsewhere in the world. As displayed earlier, regardless of the form to market (live, fresh, frozen, boiled, canned, dried etc) the abalone price "in shell" tends to equate worldwide. Of course, there will be shorter-term price variations as the economies of that handful of Asian buyer countries weakens or strengthens. In recent years, we have seen a substantial weakening of the Japanese economy—now recovering. On an equivalent yen basis, adjusted to US$, $37/kilo for a given



$20/Tin of just 252g Abalone = only $12/kilo "in shell"

Figure 4. Weight loss during canning of abalone.

Figure 5. 1999 World abalone market.

size and quality in January 1998, this would equate to $41/kilo in January of 1999 and $45/kilo in January 2000. During this same period, PR China's economy has markedly strengthened. Just a few years ago, the majority of the abalone farming production in China was designed to export this premium product to Japan and elsewhere. Today, virtually all 3,000+ tons of China's abalone production is consumed internally.

To break away from world pricing, a company responsible for large quantities of abalone, whether fished or cultured, would have some important choices to consider. These could include:

- Continue the selling pattern
- Develop premium market for export or within the exporting country
- Brand name product
- Multi functional processing and packaging
- Sous Vide and "skin packed" fresh and frozen
- Direct approaches to DWE's (distributors, wholesalers, major end users)

Continue the Selling Pattern

A company could continue along the lines of the patterns mentioned earlier (prices likened to a commodity—driven by Asian Markets—varying by species and size) which tend to equate to a similar "in shell" price worldwide.

Alternatively, a company could begin to break away from the "price equation" through implementing any one, or several, of the following choices.

Develop a Premium Market for Export and Within Currently Exporting Countries

Exporting nations should not take for granted that premium prices will continue for ever. Considerable effort will be required. You may hear "we had a strong abalone market within our country in the late 60s early 70s, however, that was when prices were very low". If we re-examine that statement on an inflation-adjusted basis, a $30/kg price today would equate to a $6.79/kg price in 1970 (Antweiler 2000). That "expensive" restaurant abalone meal at $45 today equates to a $10 "expensive" abalone meal in 1970. The premium pricing in the "home market" of the currently exporting nation will not remain as a "given". Abalone will never become a low cost food, however, effort and promotion will be needed to maintain the highest market pricing levels.

Brand Name Product

Much of the fisheries and aquaculture world has worked with brand names for many years, but this is rare in the "abalone world". Premium (higher than the most common denominator) pricing is achievable through developing a high quality, branded product and then telling the world about it.

Sous Vide and "Skin Packed" Fresh and Frozen

We live in a "food service—ready to eat—fast food" industrialized world, yet abalone traditionally goes to market in the same basic forms (live, fresh meat, frozen, canned etc.) and is distributed to the same standard markets. Larger producers should consider developing "heat and serve" processed, prepared abalone dishes. This may sound like heresy to the abalone traditionalist, but it is a clear path toward maintaining premium prices in the home markets of abalone exporting nations. Some companies have been promoting vacuum pack/skin pack for many years with rewarding results, but they represent only a small fraction of the marketplace. Fast food need not be a low-priced food. A Hong Kong food chain is currently promoting an abalone fast-food concept, with a single outlet generating over US$2,000,000 annually.

Multi Functional Processing and Packaging

Industrialized nations need to develop low cost production possibilities with highly mechanized food service facilities. (This level of processing is quite expensive, but could be accomplished on a co-op basis.) Today, several industrialized nations have such plants operating for bivalves. A modern, low-cost process complex might encompass six multifunctional production areas including raw material supply, raw material pre-treatment, processing/transformation, preservation, packing, storage.

Direct Approaches to DWE's

(Distributor, Wholesaler, and major End Users). This methodology requires considerable effort, yet is rewarding. It should be your market study that selects the DWE, not the reverse.

WORLD SUPPLY/DEMAND RELATIONSHIPS: 1977–1999–2004

Figure 6 illustrates the relationship between world supply and demand in 1975, 1999 and 2004 (predicted). In 1975 the supply was about 20,000 mt (on an adjusted basis, that might have been 24,000 mt or more). There was no major shortage of product, and this resulted in a demand/supply balance. The 1999 supply was 18,000 mt (or 13,000 mt after deducting "new market" *H. supertexta* which was not a factor in the 1975 demand) whilst the demand remained at over 20,000 mt, leaving a potential shortfall of about 7,000 mt. It is estimated that by 2004 the supply will be about 15,000 mt (after deducting "new market" *H. supertexta*) but

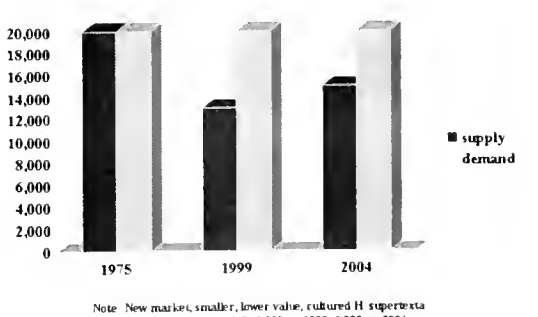


Figure 6. Abalone supply and demand in 1975, 1999 and 2004.

the potential demand is likely to remain over 20,000 mt. This will leave a potential shortfall of at least 5,000 mt.

CONCLUSIONS

World Abalone Supply

Over the past 10 years, the abalone fisheries of the world have declined by about 30% while the world's cultured abalone production has increased over 600%. The trend toward larger, cultured, premium species will continue. Ten years ago a 70 mm animal was considered "market size" whereas today market size is closer to 90 mm. Some production at 120 mm or larger will be required in the future.

Looking ahead to the year 2004, we anticipate the abalone fisheries to remain fairly flat at the 10,000 to 11,000 mt levels, while cultured abalone farms are anticipating very substantial increases. Political, environmental and pathological events will, of course, have some unknown impact on world abalone supply.

World Abalone Demand

We have been discussing a strong world demand and potential "shortfalls" but this does not necessarily imply an automatic demand at a premium price. Outside of the Asian world, the desire for this "caviar in a shell" priced animal will be directly related to the ability of the abalone industry to compete successfully in the marketplace. Among other things, this should include *Sous Vide* preparations, brand identification, sophisticated processing facilities and unique DWE programs.

For the sake of our own and future generations, we must do a better job of protecting the world's abalone fisheries, however, it is the cultured abalone industry that must not only expand production but must also increase efforts to assure a continual world premium market.

ACKNOWLEDGMENTS

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A REVIEW OF SETTLEMENT CUES FOR LARVAL ABALONE (*HALIOTIS* SPP.)

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ABSTRACT Settlement of abalone larvae involves larval attachment (a reversible behavior) followed by metamorphosis (which involves irreversible physical changes). Coralline algae induce attachment and metamorphosis in all abalone species tested, and there is some evidence of settlement preferences for certain coralline species. The settlement-inducing chemicals from corallines have not been identified. In one case (*Haliotis rufescens*) a small peptide is implicated, while in another (*Haliotis discus hannai*) halomethanes are thought to be critical. Corallines are generally regarded as unsuitable for use in hatcheries, but their potential use has not been fully evaluated. Many abalone hatcheries rely on biofilms to induce larval settlement. The activity of biofilms increases with their age. Ungrazed films are generally dominated by fast-growing benthic diatoms, and settlement on these films is variable and often low. Few diatom strains are consistently good for settlement, and strains that are excessively mobile, or form 3-dimensional colonies, can prevent successful settlement. The chemistry of settlement induction by biofilms, and the role that bacteria play, are poorly understood. Bacteria can induce settlement, but metamorphosis occurs gradually over several days, if at all. Pregrazing by conspecific abalone improves the settlement-inducing activity of a biofilm. The mucus from the foot of grazers probably contains chemicals (not identified) that trigger attachment or metamorphosis, particularly when combined with another cue (such as dibromomethane or a biofilm). Various pure chemicals induce attachment and/or metamorphosis of abalone larvae. These may bind to larval receptors (e.g., γ-aminobutyric acid = GABA) or act "downstream" of, or "parallel to", the receptors (e.g., compounds that depolarize membranes or alter levels of cyclic AMP or calcium). None of these chemicals is considered to be a natural settlement cue, and only GABA has been used in abalone hatcheries. The timing and end point of the abalone settlement response vary among cues, and among abalone species. Cues can combine synergistically to enhance settlement (e.g., GABA with lysine, biofilm or coralline extract, dibromomethane with mucus). Signal reception pathways are known only for the case of *Haliotis rufescens* and GABA-mimetic inducers. The response is controlled through a "morphogenetic pathway", which is subject to up- and down-regulation by molecules acting on a separate "regulatory pathway". Larval attachment and metamorphosis are often uncoupled in abalone. Cues for attachment are much more widespread than cues for metamorphosis.

KEY WORDS: abalone, attachment, metamorphosis, diatoms, biofilms, coralline algae, settlement cues

INTRODUCTION

Abalone larvae swim for several days before becoming competent to undergo settlement, which transforms them into a crawling, benthic snail. Settlement is triggered by external cues. Knowledge of these cues is critical in abalone culture where complete, rapid and predictable settlement is desired but seldom achieved (Searcy-Bernal et al. 1992a, Roberts et al. 1998). Settlement cues also play a role in determining where abalone recruit, and knowledge of cues may be useful in site choice for larval reseeding.

Several authors have previously reviewed aspects of abalone larval settlement. The extensive work by Daniel Morse and colleagues on metamorphosis induction in *Haliotis rufescens* has been reviewed in detail (A. Morse 1991, D. Morse 1992). McShane's (1992, 1995) reviews discuss settlement in natural habitats and attempt to draw links between laboratory and field data. Hahn (1989c) presented a detailed summary of the data on abalone settlement induction as of 1985. In the decade or so since these reviews there has been substantial research on abalone larval settlement. The new work has broadened the range of abalone species studied from its early focus on *Haliotis rufescens* and *Haliotis discus hannai*, and has also broadened the range of cues that have been addressed, and the depth of investigation for certain key inducers. The body of data now available is adequate to justify a comprehensive review. This study concentrates on abalone larval settlement cues, whether natural or otherwise. Ecological relevance is discussed only briefly. This review attempts to emphasize the distinction between the components of the settlement response, which has received little critical attention in previous reviews. It also attempts to highlight similarities and differences among abalone species. Both of these tasks were made difficult by varying methodology and terminology, which are discussed in detail at the outset in the hope that greater standardization will result. The review concludes by summarizing some major points arising from the review, making some comments on settlement cues for hatcheries, and providing some suggestions about priorities for future research in this field.

SETTLEMENT TERMINOLOGY AND METHODOLOGY

Terms Used to Describe Settlement Stages

Various terms have been used interchangeably in publications dealing with abalone larval settlement (Table 1) so clarification is required. In this review, the transition from a swimming larva to a crawling, feeding post-larva is divided into three stages. The first is larval "attachment"—the larvae stop swimming, sink to the bottom, and attach to the substratum by their foot. Larvae retain their velum during attachment and some larvae choose to resume swimming—attachment is a reversible behavior. The next stage marks the initiation of metamorphosis and is conveniently indicated by the shedding of the larval velum, committing the abalone to benthic life. This stage is here termed "metamorphosis", and involves an array of irreversible physical changes. If the post-larva continues to develop normally for a day or two after initiation of metamorphosis, then the mouth will open, feeding will begin, and flared peristomal shell will begin to grow. This further metamorphic development is sometimes separated from the earlier shedding of the velum, and is referred to here as "shell growth". The term settlement is used as a general term for the transition from a larva to a post-larva. Data that do not distinguish between attachment and metamorphosis are reported here as "settlement" (in quotation marks).

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TABLE 1.

Examples of terms used in published abalone settlement studies and the corresponding term used in this review.

Term Used in Original Paper	Equivalent Term in this Review	Notes	Reference
Settlement	Attachment + metamorphosis	Settlement defined as being animals that had shed the velum. Attachment without metamorphosis was not quantified.	Daume et al. (1999a, 1999b, 2000)
Settlement	Attachment	Attachment scored separately from metamorphosis.	Morse et al. (1979b) Kawamura & Kikuchi (1992) Searcy-Bernal et al. (1992) Slattery (1992), Seki (1997)
Inferred settlement	Unknown	Calculated from number of larvae still swimming. "Settled" larvae would include those attached or metamorphosed plus any stuck to surfaces.	Moss (1999)
"Settlement"	Unknown	Paper does not define settlement.	Genade et al. (1988)
Behavioural metamorphosis	Attachment		Morse et al. (1980a)

Settling abalone larvae often do not progress rapidly through attachment, metamorphosis and shell growth. Larval attachment is not a useful end point for an abalone larva, or an abalone farmer, so the distinction between the stages of settlement is critical in interpreting data. It is difficult to make these distinctions in the hatchery, and even more so in the sea, so laboratory experiments have proven very useful in the study of abalone settlement cues.

Abalone Settlement Assay Methods

In static settlement bioassays the use of antibiotics to suppress bacterial growth is essential for some abalone species to ensure consistently high larval survival (Morse et al. 1979b). Different degrees of bacterial suppression led to conflicting results over the activity of γ-aminobutyric acid (GABA) (Morse 1992). Negative controls are crucial to ensure that settlement responses are not falsely attributed to particular cues. Several studies have reported high attachment or "settlement" in negative controls (Liu et al. 1986, Genade et al. 1988, Slattery 1992, Moss 1999) making it difficult to interpret settlement induction by other treatments. Some problems with antibiotic toxicity and interference have been reported. Streptomycin was toxic to *Haliotis diversicolor* at doses as low as 5 $\mu g\ ml^{-1}$ (Bryan & Qian 1998) and emetine toxicity caused abnormal loss of velum cells in *Haliotis rufescens* (Fenteany & Morse 1993). Anisomycin caused slight attachment induction in *Haliotis rufescens*, but also stimulation of swimming activity that partly antagonized the response to GABA (Fenteany & Morse 1993).

The methods used will depend on the objectives of an experiment. If the objective is to determine larval responses in hatchery conditions then full-scale hatchery systems should be used and antibiotics will be inappropriate. However, if the experiment is to determine the settlement response of larvae to particular cues then the assay system should be designed to eliminate all potentially confounding influences, including bacterial interference.

If larval mortality or morbidity in assays complicates interpretation then this needs to be discussed. For example, Moss (1999) recorded high "inferred settlement" by day five of an assay but then explained that mortalities were very high (88 to 97% by day seven) making interpretation of "settlement" data difficult. Larval and post-larval abalone have considerable ability to survive without particulate food (Searcy-Bernal 1999, Takami et al. 2000, Roberts & Lapworth 2001, Roberts et al. 2001). This suggests that high mortality within a few days of settlement induction (Daume et al. 1999b, Moss 1999) is likely to be caused by factors other than starvation. Dead or moribund larvae should be excluded from calculation of percentage settlement, but they still introduce uncertainty. Would those animals have settled if they were healthy? Is the larval settlement response influenced by the marginal conditions in the assay?

Similar attention needs to be paid to toxic responses that can cause velum loss and could be confused with the onset of metamorphosis. Typical symptoms of toxicity include partial loss of the velum, an increasing number of larvae found lying on their sides (rather than attached by the foot or swimming high in the water column) and lack of shell growth (Fenteany & Morse 1993).

Assay counts may need to include abalone on the walls of assay chambers (Genade et al. 1988) or on the surface film of the assay water (Roberts & Nicholson 1997). The difficulty of accurately counting swimming larvae has been circumvented by doing retrospective destructive counts (e.g. Roberts & Nicholson 1997) or removing the settlement substrate to aid counting (e.g., Moss 1999).

While larval attachment is generally rapid, many cues cause only a gradual rise in the percentage metamorphosis over a week or more (e.g. Kawamura & Kikuchi 1992, Roberts & Nicholson 1997). Thus, the period of observation influences the settlement observed, and this must be taken into account when comparing studies. The timing of observations should be tailored to the question being addressed, and multiple observations will be most informative.

Larval age can affect settlement responses. Abalone larvae have a pre-competent phase of several days during which they will not respond to settlement cues. Various morphological and behavioral features have been used to indicate competence to settle. These include the appearance of the fourth tubule on the cephalic tentacles (Seki & Kan-no 1977, Ebert & Houk 1984), exploratory

crawling (Seki & Kan-no 1977) and the presence of at least three rows of chitinized radula teeth (Moss & Tong 1992a). Morse et al. (1979b) used a 30–120 minute exposure to GABA to test larval competence to attach. An indirect method is to calculate larval stage based on larval age, rearing temperature and biological zero point (Seki & Kan-no 1977, Hahn 1989b). Larvae of many abalone species reach competence to settle after several days at 15 to 20° C (Hahn 1989b). The ability to attach arises earlier than the ability to metamorphose, and the speed of a settlement response can vary with larval age (e.g., Morse 1984a, Barlow 1990). *Haliotis diversicolor* larvae can begin attaching as early as 12 hours after fertilization (at 24 C) but may not metamorphose until several days later (Liu et al. 1986, Bryan & Qian 1998). Positive controls should be included in experiments to confirm larval competence. Data can even be normalized to a positive control to take account of inter-batch variability in larval responsiveness (Baloun & Morse 1984).

Other factors that may affect the outcome of abalone larval settlement assays include: (1) the use of vertical versus horizontal surfaces (Bryan & Qian 1998), (2) the size of the settlement substratum relative to the assay chamber (e.g., Daume et al. 1999a, Daume et al. 1999b versus Kawamura & Kikuchi 1992), (3) the increased sensitivity of older larvae to settlement cues (Barlow 1990, Moss & Tong 1992a, Yang & Wu 1995), (4) variability between batches of larvae (e.g., Baloun & Morse 1984, Trapido-Rosenthal & Morse 1986b, Roberts & Nicholson 1997), and (5) changes in light intensity causing attached larvae to resume swimming (Moss & Tong 1992a, Moss 1999).

CORALLINE ALGAE AS SETTLEMENT CUES

The Corallinaceae are divided into articulated (= geniculate) and non-articulated (= non-geniculate) forms (Johansen 1981). The non-articulated corallines are commonly referred to in the abalone literature as crustose corallines, so this review uses the terms crustose and articulated corallines. The crustose corallines occur in a wide range of growth forms (Woelkerling et al. 1993, Shepherd & Daume 1996).

In the wild, juvenile abalone are closely associated with crustose coralline algae (e.g., Morse et al. 1980c, Shepherd & Turner 1985, McShane & Smith 1988, Shepherd & Daume 1996, Day & Branch 2000). Laboratory experiments show that intact crustose coralline algae are among the best inducers of larval attachment and metamorphosis for all abalone species tested (Morse et al. 1980c, Morse & Morse 1984a, Moss & Tong 1992a, Roberts & Nicholson 1997, Takami et al. 1997, Daume et al. 1999a, Daume et al. 1999b, Moss 1999). Crustose coralline algae generally induce strong and rapid metamorphosis (>80% within two days) but *Haliotis rubra* and *Haliotis laevigata* sometimes show much weaker responses to three species of crustose corallines (Daume et al. 1999b, Daume et al. 2000).

Articulated corallines were much less effective (<10% attachment) than crustose corallines as settlement cues for *Haliotis rufescens* (Table 2, Morse et al. 1980c, Morse & Morse 1984a). The articulated *Corallina officinalis* showed moderate to strong activity with *Haliotis virginea* but was less effective than crustose corallines (Table 2). Settlement of *Haliotis cyclobates* on sea grasses may be triggered by the crustose corallines that cover 13–20% of the seagrass blades, or by other cues such as the sea grass or its surface biofilm (Stevenson & Melville 1999).

When given a choice of crustose coralline species larvae of *Haliotis laevigata* selected *Sporolithon durum* in preference to *Mesophyllum engelhartii* or *Hydrolithon rupestre* (Daume et al. 1999a). This is consistent with the disproportionately high number of *Haliotis laevigata* recruits found on *Sporolithon durum* in the wild (Shepherd & Daume 1996).

OTHER MACRO-ALGAE AND BLUE-GREEN ALGAE

Crustose red algae are the only seaweeds known to strongly induce *Haliotis rufescens* settlement while intact. This observation is based on a relatively small sample of "other" seaweed species (Table 2). Larval attachment was ≤1% after 24 hours of exposure to intact foliose algae or blue-green algae (Morse et al. 1980c, Morse & Morse 1984a, Morse et al. 1984). *Haliotis discus hannai* seems to be less discriminatory—Seki (1997) found that 6 out of 12 foliose macro-algae induced metamorphosis of *Haliotis discus hannai* larvae. The active species included brown, red and green algae (Table 2).

Ulvella lens is a small, crustose green alga that grows as prostrate rosettes on hard surfaces. It is common on heavily grazed surfaces such as abalone tanks, and has been used in commercial abalone hatcheries as a settlement cue. Pregrazed films of *Ulvella* produced >90% attachment (Takahashi & Koganezawa 1988, Seki 1997) and metamorphosis (Takahashi & Koganezawa 1988) of *Haliotis discus hannai* larvae while ungrazed *Ulvella* films induced 60–70% attachment and metamorphosis (Takahashi & Koganezawa 1988). Germlings of *Ulvella lens* induced 10–50% of *Haliotis rubra* larvae to metamorphose within one to three days (Daume et al. 2000). This was higher than the metamorphosis recorded on films of several benthic diatom species in that study.

Prostrate brown algae of the genus *Myrionema* are often abundant (along with *Cocconeis* spp. and *Ulvella lens*) on the pregrazed plates used successfully to settle abalone (Seki 1980, Suzuki et al. 1987). The author is not aware of any data on the settlement-inducing activity of *Myrionema* spp.

CHEMICALS EXTRACTED FROM ALGAE AND BLUE-GREEN ALGAE

Attempts to purify the chemicals that trigger abalone settlement have focused on crustose coralline algae because of the corallines' consistently strong activity and their role in abalone ecology. The extensive work on purifying chemical inducers of *Haliotis rufescens* settlement has been reviewed previously (A. Morse 1991, D. Morse 1985, D. Morse 1990, D. Morse 1992), so only a brief account is given here. When intact seaweed specimens were tested, only crustose red algae induced high levels of larval attachment and metamorphosis of *Haliotis rufescens* larvae (Table 2). However, testing of crude extracts revealed that blue-green algae and foliose red algae also contained inducers intracellularly, while two brown algae, a green alga and a bacterium did not (Table 2, Morse & Morse 1984a, Morse et al. 1984). Red and blue-green algae contain phycobiliproteins, which the other algae lack (Morse 1985). The activity from coralline *Lithothamnium californicum*, the foliose red alga *Porphyra* sp., and the blue-green alga *Spirulina platensis* was initially protein-associated, but could be chromatographically separated from proteins into a low molecular weight (~650–1250 *Mr*) form (Morse & Morse 1984a, b, Morse et al. 1984, Morse 1992). The activity of the protein-associated form was enhanced by boiling, proteolytic digestion, and acid hydrolysis, suggesting that the active product may have been an amino acid or small peptide (Morse et al. 1979a). The peptide-associated

TABLE 2.

Settlement of abalone larvae in response to macroalgae and blue-green algae.

Algal Species	*Haliotis rufescens* Attachment & Metamorphosis	*H. rufescens* No. of Algal Species	*H. virginea* % Attach. (Range of means or Mean ± SE)	*H. virginea* % Met. (Range of means or Mean ± SE)	*H. virginea* No. of Algal Species	*H. discus hannai* Attachment & Metamorphosis	*H. discus hannai* No. of Algal Species
Intact algae			2 day	2 day			
Crustose coralline	+	4	84–95	64–88	4	+	1
Articulated coralline	+(≤8%)	2	89 ± 2	56 ± 5	1		1
Crustose non-coralline red	+	1					
Foliose red	–	3				+/–	(3/2)
Foliose brown	–	2				+/–	(2/4)
Foliose green (*Ulva* spp.)	–	1				+	1
Crustose green (*Ulvella* lens)						+	1
Blue-green	–	1					
Crude extract from:			Dose of 20 μg protein ml^{-1}				
			2.5 day	5.5 day			
Crustose coralline	+	4	56–81	18–65	3		
Articulated coralline	+	2	57 ± 6	68 ± 13	1		
Crustose non-coralline red	+	1	38 ± 5	63 ± 8	1		
Foliose red	+	3	88 ± 9	Toxic	1		
Foliose green	–	1	10 ± 4	4 ± 0	1		
Foliose brown	–	2	18–38	2–13	2		
Blue-green	+	3	21 ± 2	6 ± 1	1		
Filtered seawater control			6 ± 5	0 ± 0			
0.01 M Tris buffer control			1 ± 0	0 ± 0			
References	Morse and Morse (1984a) Morse et al. (1984)		R. D. Roberts unpubl. data[a]			Seki (1997)	

[a] To prepare crude extracts, 4 g of fresh alga (or 1.5 g dry *Spirulina*) was disrupted with a Bellentini bead shaker in ~25 ml 0.01 M Tris-HCl (pH 7.0) then centrifuged at 24,000 × g for 10 min at 2°C. Protein content of the supernatant (= crude extract) was determined by the Lowry method and extracts were assayed as per Roberts and Nicholson (1997). Data presented are all for a dose of 20 μg protein ml^{-1} of assay medium. Greater doses generally gave higher attachment and metamorphosis, but often became mildly toxic to larvae within a few days.

Note: "Attach." = attachment. "Met." = metamorphosis. To read *Haliotis discus hannai* data: "+/– (3/2)" means induction by 3 species of foliose reds and lack of induction by 2.

inducer molecule is known to contain several unidentified unusual amino acids but its precise structure is still unknown (Morse 1992). The inducer purified from *Lithothamnium* mimics the activity of GABA.

The response of *Haliotis virginea* to algal extracts is complex. At a low dose, the activity of extracts from various algal groups (Table 2) appears similar to that reported for *Haliotis rufescens*, except that blue-green algae have low activity. At higher doses, the brown algae induced means of 72–76% larval attachment within two days but caused mild toxicity after five days (data not shown). While most extracts induced some degree of larval attachment quite rapidly, metamorphosis occurred only gradually (Table 2) and shell growth was almost completely inhibited. A similar response to coralline extracts (Fig. 1) has been reported previously for both *Haliotis virginea* and *Haliotis iris* (Roberts and Nicholson 1997). The lack of shell growth appears to result from toxicity of co-extracted compounds preventing normal post-larval development and activity subsequent to the shedding of the velum (Roberts and Nicholson 1997). Extracts from the coralline alga *Phymatolithon repandum* contained inducers in both low molecular weight (600–1100 *Mr*) and relatively high molecular weight (>10,000 *Mr*) fractions of polar extracts (Roberts et al. 1994), paralleling the situation with *Haliotis rufescens* and *Lithothamnium californicum* (Morse et al. 1984). The active chemicals have not been identified.

Attempts to purify chemical inducers of urchin metamorphosis from coralline algae (Taniguchi et al. 1994) led to the discovery that metamorphosis of *Haliotis discus hannai* larvae is induced by a combination of dibromomethane and mucus (Seki et al. 1997). Larval attachment and metamorphosis on corallines are hypothesized to be independently triggered by trail mucus and dibromomethane respectively (Seki et al. 1997). Dibromomethane is a volatile chemical naturally released from corallines and many other algae (Taniguchi et al. 1994).

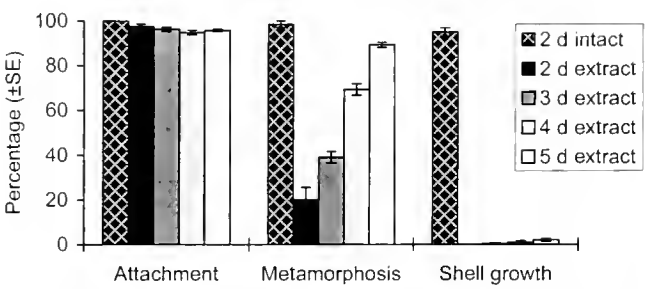

Figure 1. Comparison of the settlement response of larval *Haliotis iris* to intact plants and aqueous extract (440 μg dry extract ml^{-1}) of the coralline alga *Phymatolithon repandum*. Intact plants induced rapid attachment, metamorphosis and shell growth, whereas extracts induced rapid attachment, gradual metamorphosis and negligible shell growth. Data are from Roberts and Nicholson (1997).

BIOFILMS (INCLUDING BENTHIC DIATOMS)

Biofilm Composition

A biofilm is a layer of microorganisms and organic matter that develops on aquatic surfaces. Biofilms have long been used to induce larval settlement in abalone hatcheries around the world. Biofilms that develop in flowing seawater with light are often dominated by diatoms. The succession that occurs in the diatom community is important in abalone farming. The film is initially comprised largely of fast-growing, prostrate, benthic diatoms (Kawamura 1995). Over time, diatoms that grow as upright colonies (often referred to as 3-dimensional, overstorey or filamentous diatoms) grow above the prostrate forms (Kawamura 1995). Grazing by juvenile or adult gastropods removes the overstorey diatoms (Types C-H in Fig. 2), and loosely attached prostrate forms (Type A in Fig. 2), so only grazing-resistant, prostrate algae remain (Seki 1980, Suzuki et al. 1987, Kawamura 1995, Matthews and Cook 1995). The grazed (or secondary) film is often dominated by diatoms of the genus *Cocconeis* often along with small prostrate macroalgae such as *Ulvella lens* and *Myrionema* spp. (Suzuki et al. 1987, Seki 1980, 1997).

Use of Biofilms in Abalone Hatcheries

Some early abalone culture cued settlement with natural biofilms, without exerting any control over algal composition (Grant 1981, Chen 1989). The observation that filamentous diatoms were unsuitable for settlement (Seki 1980) led to the use of pre-grazing to select for tightly attached, prostrate diatoms (Type B in Fig. 2) and other algae (Seki 1980). Pregrazed films became the settlement method of choice in Japan.

Some other abalone hatcheries chose to select for small, prostrate diatoms by using filtered water to condition settlement surfaces, or by filtering diatom slurries prior to inoculation of tanks. An ungrazed film dominated by small, prostrate diatoms (Type A in Fig. 2) was the standard settlement preparation in many countries including Australia (Hone et al. 1997), Korea (Shallow Seafarming Research Institute 1990), New Zealand (Tong et al. 1992), and the USA (Ebert & Houk 1984).

Greater control of the diatom species in the film is introduced by isolating particular strains and growing them in monocultures (e.g., Hahn 1989a, Tong & Moss 1992, Kawamura & Kikuchi 1992). However, knowledge of the dominant diatom species still provides limited description of the characteristics of the biofilm. In addition to diatoms, a biofilm will contain many strains of bacteria, fungi and other microbes, as well as a variable amount of extracellular secretions, and a wide array of organic molecules. All of these components will change as the film develops. Given this complexity, it is perhaps not surprising that we still do not know which characteristics of a biofilm are responsible for settlement induction.

Settlement Versus Biofilm Age and Source-Habitat

Older biofilms appear to be better than younger ones for abalone larval settlement. The percentage of *Haliotis iris* larvae that settled on biofilms developed in flowing seawater increased with the age of the film (Moss & Tong 1992b). In this case maximum film age was nine days, and mean settlement was ~22% after six hours. A similar pattern was found with *Haliotis virginea* larvae—films grown for 36 days induced more attachment (77 ± 9%) and metamorphosis (25 ± 12%) within three days than films grown for 1, 4, or 12 days before assayed (Roberts et al. 1997b). This pattern with respect to biofilm age is reinforced by data from specific diatom strains reviewed below.

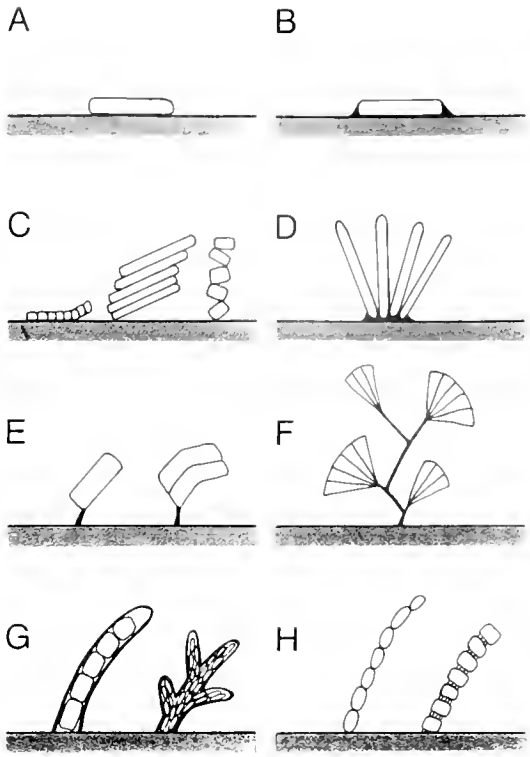

Figure 2. Schematic representation of eight growth forms of benthic diatom (Kawamura 1994). *Type A* (gliding prostrate type). Solitary cells with a prostrate form and swift gliding movements. Adhesive strength is very low. *Type B* (adhesive prostrate type). Solitary cells with a prostrate form, slow movement, and high adhesive strength. *Type C* (non-motile upright type). Non-motile solitary cells or simple, fan-shaped colonies standing upright with a relatively weak adhesion to the substratum. *Type D* (belt-shaped colonial type). Long, belt-shaped or zigzag colonies attached strongly to the substratum by a terminal cell of the colony. *Type E* (mucous thread solitary type). Solitary cells or short, belt shaped colonies attached strongly to the substratum with a mucous thread. *Type F* (mucous thread colonial type). Arborescent colonies connected with mucous threads and attached strongly to the substratum by a single mucous thread. *Type G* (tube-dwelling colonial type). Colony cells are enclosed in a filamentous or arborescent branched mucous tube. Cells can move within the tube, and the tube is tightly attached to the substratum. *Type H* (filamentous colonial type). Filamentous colonies attached only weakly to the substratum by a terminal cell of the colony.

The settlement inducing activity of biofilms is not limited to films from suitable abalone habitats. Biofilms grown in unsuitable habitats (rivers and estuaries) induced more attachment (site means ± s.e. of up to 83 ± 5 and 72 ± 5% respectively) than negative controls (6 ± 2%) after three to four days (Roberts et al. 1997b).

Diatoms

Diatom films have long been used to induce larval settlement in abalone hatcheries around the world. The species composition and other biofilm characteristics are often not controlled or recorded. Only a handful of studies have examined the settlement of abalone larvae in response to specific diatom strains. The picture they give

is one of variability and complexity that highlights the limited knowledge of settlement induction by diatoms. Some of the physical features of diatoms that affect their settlement-inducing activity are known (see later) but these explain relatively little of the variation seen among strains. It is likely that details of biofilm chemistry will explain settlement patterns, so detailed study of biofilm chemistry will be needed to significantly advance our understanding.

Features Making Diatom Films Favorable or Unfavorable for Settlement

Kawamura and Kikuchi (1992) quantified larval attachment, metamorphosis, shell growth and survival over two weeks of exposure to various densities of 22 diatom strains. Data representative of the major patterns of response are shown in Fig. 3. All strains induced over 70% attachment within two days of exposure, but few strains induced rapid and complete metamorphosis. Diatoms that induced relatively rapid metamorphosis were non-colonial, prostrate forms (Types A and B in Fig. 2), but many diatoms of this growth form did not give strong metamorphosis. At high densities, diatoms with three-dimensional growth forms entangled larvae and prevented them from metamorphosing. At low densities these diatoms induced gradual attachment and metamorphosis, as seen with many prostrate diatoms. An English summary of this study was presented in Kawamura (1996).

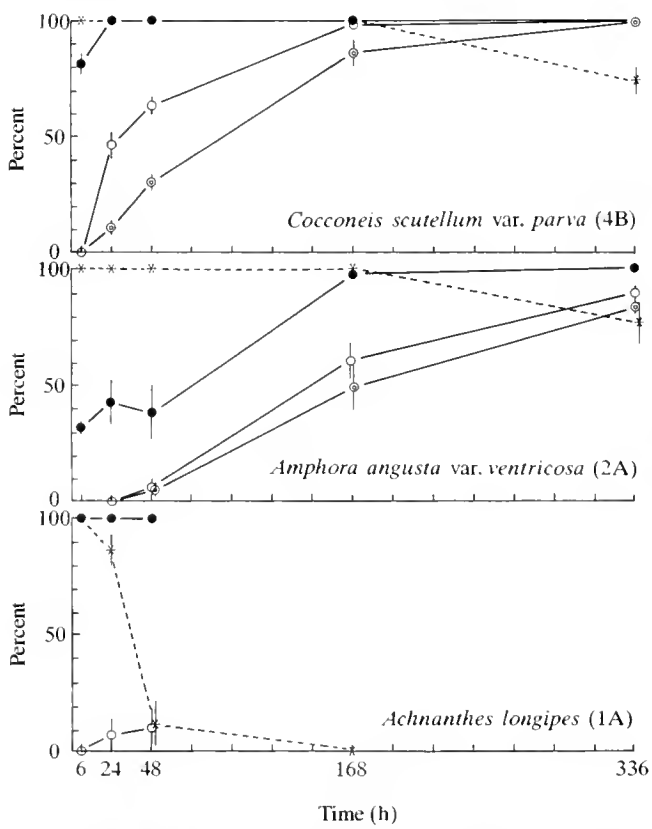

Figure 3. Examples of three patterns of response observed for *Haliotis discus hannai* larvae settling on benthic diatoms. Data (mean ± s.e.) show changes in attachment (— ● —), metamorphosis (— ○ —), shell growth (— ⊡ —) and survival (— * —) over 2 weeks of continuous exposure to three diatom treatments, 4B, 2A and 1A (Kawamura and Kikuchi 1992).

For prostrate diatoms, there is generally a positive relationship between diatom density and abalone larval settlement. This relationship is generally apparent for a given diatom strain within an experiment (Kawamura & Kikuchi 1992, Daume et al. 1999a) but does not necessarily apply across diatom species or across experiments. Larval attachment of *Haliotis discus hannai* within six hours was low when diatom density was less than 1×10^4 cells.cm^{-2}, and high mainly for diatom densities above 3×10^4 cells.cm^{-2} (Kawamura and Kikuchi 1992). Given that the size of diatom cells varies considerably, percent cover may be more useful than density as a measure of diatom abundance.

The abundance of a diatom film may be important in providing sufficient inducer to trigger larval attachment. As diatom films develop and age, they go through a series of growth phases (O'Meley & Daintith 1993) and many physical and chemical characteristics change along the way. It may be one or more of these factors that causes increased settlement rather than diatom abundance *per se*.

Diatom Species Favorable and Unfavorable for Settlement

It is not yet possible to list diatom strains that are consistently good or bad for abalone larval settlement. *Cocconeis* spp. have gained a reputation of being good for settlement because diatoms of this genus are often dominant on the pre-grazed plates that induce settlement in Japanese abalone hatcheries (Akashige et al. 1981, Suzuki et al. 1987, Seki 1997). Testing of pure strains reveals a more complex picture. *Cocconeis scutellum* is often effective as a metamorphosis inducer, although metamorphosis sometimes occurs gradually over several days (Kawamura & Kikuchi 1992, Roberts & Nicholson 1997). Daume et al. (1999b) reported only 10–30% metamorphosis of *Haliotis laevigata* larvae in response to *Cocconeis scutellum*. However, if Daume et al.'s much shorter observation period (24 hours) and lower diatom densities are taken into account, the results are comparable to those obtained with *Haliotis discus hannai* (Kawamura & Kikuchi 1992) and *Haliotis virginea* (Roberts & Nicholson 1997). Ohgai et al. (1991) found that settlement on *Cocconeis* sp. was 12 times higher than on *Nitzschia closterium* or a mixed biofilm of naturally seeded diatoms, but only 1.5 times higher than on *Navicula ramosissima*.

Cocconeis strains are not always good for abalone settlement induction. Induction of metamorphosis can be slow (see above) and some strains of *Cocconeis* have given poor settlement induction (Ishida et al. 1995). *Haliotis rubra* larvae did not respond within 24 hours to *Cocconeis scutellum* or any of the four other diatom strains tested (Daume et al. 2000). Mean metamorphosis within 24 hours was ≤6% in all cases. Similarly, less than 2% of *Haliotis rubra* larvae metamorphosed within three days on four diatom strains (6-day-old film, diatom densities not presented), while up to 25% of larvae settled on the coralline alga *Phymatolithon repandum* (Daume et al. 1999b). In equivalent bioassays, *Haliotis laevigata* larvae responded strongly to the same four diatom strains (Daume et al. 1999b) implying a major difference in the settlement requirements (or speed of response) between these two Australian abalone species.

Cylindrotheca closterium (and the physically similar *Nitzschia longissima*) are often poor inducers of attachment and metamorphosis, perhaps because of their high mobility and low attachment strength (Ohgai et al. 1991, Kawamura & Kikuchi 1992, Roberts et al. 1997a). However, *Cylindrotheca closterium* has on occasion strongly induced metamorphosis of *Haliotis discus hannai* larvae

(Kawamura & Takami 1995) and *Haliotis laevigata* (Daume et al. 1999b).

BACTERIA

Bacteria are ubiquitous in so-called "diatom" films, yet the role that they play in settlement induction is virtually unstudied. Roberts et al. (1997a) found that metamorphosis of *Haliotis iris* larvae was reduced by 60% if the inducing diatom (*Nitzschia ovalis*) was grown in the presence of antibiotics. The proportion of larvae that attached was similar between the treatments. No work has been done on settlement induction by axenic diatoms, so it is not certain that diatoms can induce abalone settlement without bacteria present. The fact that many diatom strains are active when assayed in the presence of antibiotics does not preclude (1) bacterial production of inducers prior to bioassay; or (2) activity resulting from bacteria unaffected by the antibiotic treatment. It is practical to obtain axenic diatom cultures, but keeping them axenic during a larval assay would be far more challenging. Detection of differences resulting from antibiotic treatments (e.g., Johnson & Sutton 1994) or near-axenic treatments would suggest a role for bacteria, but negative results would be equivocal.

The limited work done on abalone settlement induction by bacteria shows that bacteria can induce larval attachment and metamorphosis in some abalone species, but that settlement is slow. *Haliotis virginea* larvae incubated with a natural bacterial assemblage, took a week to reach 50% metamorphosis, and two weeks to reach 80% metamorphosis. Parallel assays with antibiotics retained low bacterial densities and had less than 2% metamorphosis over the same period (Roberts et al. 1997b). Similarly, gradual attachment and metamorphosis has been recorded with *Haliotis iris* exposed to bacteria in static assays without antibiotics (R. D. Roberts unpubl. data). Morse et al. (1979b) discussed gradual and inefficient attachment and metamorphosis of *Haliotis rufescens* larvae occurring in parallel with rising mortality and microbial growth. They suggested that such responses may reflect the production of GABA or the less active glutamate by bacterial metabolism. Bryan and Qian (1998) reported 20–50% attachment of *Haliotis diversicolor* within 24 hours on three bacterial strains isolated from a diatom film. Assays were conducted without antibiotics, and metamorphosis data were not reported. None of 33 strains of marine bacteria induced settlement in *Haliotis rufescens* (A. Morse's unpubl. data cited in Morse 1992, methods not presented).

PREGRAZED FILMS AND ABALONE MUCUS

Algal biofilms grazed by juvenile or adult abalone are widely effective as inducers of abalone larval attachment and metamorphosis (Takahashi & Koganezawa 1988, Ishida et al. 1995, Searcy-Bernal et al. 1992b, Slattery 1992, Conroy et al. 1996, Seki 1997, Bryan & Qian 1998, Daume et al. 2000). Where comparisons have been made between grazed and ungrazed films, the grazed films have performed better for both diatom films (Slattery 1992, Searcy-Bernal et al. 1992b, Seki 1997, Bryan & Qian 1998) and *Ulvella lens* (Takahashi & Koganezawa 1988, Daume et al. 2000).

There are varying reports on the activity of abalone trail mucus as a settlement inducer. Conspecific mucus alone was reported to induce only larval attachment in *Haliotis discus hannai* (Seki et al. 1997). In contrast, Yang and Wu (1995) found that mucus induced over 90% metamorphosis and shell growth of *Haliotis discus hannai* larvae within 48 hours. In Yang and Wu's study the mucus was laid down and assayed in the absence of antibiotics. In *Haliotis iris* mucus alone induced only a low percentage of attachment and metamorphosis when assayed in the presence of antibiotics (Fig. 4). For *Haliotis diversicolor* adult mucus alone induced moderate levels of larval attachment within six hours of exposure, but attachment subsequently fell as high mortality developed (Bryan & Qian 1998). Metamorphosis data were not presented. Morse et al. (1980c) recorded no settlement of *Haliotis rufescens* larvae in response to juvenile conspecifics (that would have produced trails of mucus).

The induction of *Haliotis discus hannai* larval attachment by trail mucus appears to be genus-specific. Larval attachment was induced by trail mucus from four Japanese abalone species but not by trail mucus from three other gastropods (Seki & Kan-no 1981b, see Seki & Taniguchi 1996 for a summary in English). The mucus left by abalone during grazing gave higher larval attachment than that left by crawling abalone, while mucus from abalone pressed or rubbed against settlement plates did not induce larval attachment (Seki & Kan-no 1981b, Seki & Taniguchi 1996). This pattern was hypothesized to result from differences in the physical or chemical properties of mucus produced during different activities (Seki & Kan-no 1981b). The presence of remnants of grazed biofilm in the grazing mucus treatment raises possible alternatives: (1) release of chemicals by grazing damage to biofilm organisms; (2) interaction between mucus and biofilm chemicals; (3) microbial proliferation on the trail mucus providing settlement cues.

The chemical basis of settlement induction by trail mucus or pre-grazed films has not been studied. Rodriguez et al. (1993) suggested that heparin-binding growth factors might be the substance responsible for settlement induction by abalone mucus, but there is no evidence to support this hypothesis.

PURE CHEMICALS

The Morphogenetic and Regulatory Signal Transduction Pathways

Before discussing pure substances that can trigger larval attachment and/or metamorphosis, it is necessary to briefly describe current understanding of the way in which inducer molecules bring about a response in larval abalone. This knowledge is based on

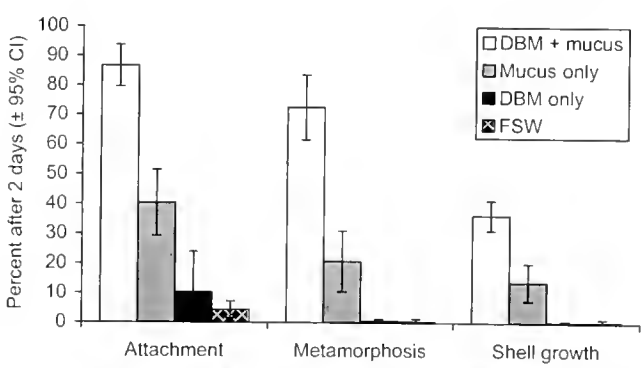

Figure 4. Synergy between dibromomethane (DBM) and mucus in settlement induction of *Haliotis iris* larvae. One juvenile *Haliotis iris* (~10 mm shell length) was held overnight in each well of a new multiwell plate (Falcon 3043), then removed before addition of 3 ml of 0.2 µm-filtered seawater, antibiotics (150 µg ml^{-1} penicillin + streptomycin sulfate), dibromomethane (175-700 µg ml^{-1}, no dose-response within this range) and 50-100 larvae. Counts were made after 48 hours of incubation (dark, 17±0.5 C). FSW = filtered seawater. R. D. Roberts (unpubl. data).

extensive research with *Haliotis rufescens* (previously reviewed by A. Morse 1991, D. Morse 1984b, 1985, 1990, 1992) and the degree to which it applies to other abalone is not known.

A simplified working model describes two interacting biochemical signal pathways—the morphogenetic and regulatory pathways. These pathways are well summarized and illustrated by Morse (1991). The morphogenetic pathway describes how an external chemical signal is translated into a nerve impulse. The model hypothesizes that binding of a GABA-mimetic molecule at stereochemically-specific receptors activates adenyl cyclase enzyme that catalyzes the synthesis of cyclic adenosine-monophosphate (AMP) as a secondary messenger. The cyclic AMP activates a protein kinase that phosphorylates an unknown protein. This protein somehow causes opening of an anion channel in the chemosensory cell membrane allowing efflux of anions and causing depolarization of the cell. This depolarization provides an electrochemical signal that can be transmitted by the larval nervous system (A. Morse 1991, D. Morse 1992). This nerve impulse can then trigger larval responses such as cessation of swimming, foot exploration behavior, and metamorphic changes in gene expression and morphology (Cariolou & Morse 1988, Barlow 1990, Groppe & Morse 1993, 1994, Degnan & Morse 1995, Degnan et al. 1995, 1997).

The larval response to GABA and GABA-like molecules (via the morphogenetic pathway) is subject to up- and down-regulation via a separate regulatory pathway (Trapido-Rosenthal & Morse 1985, Trapido-Rosenthal & Morse 1986a, Trapido-Rosenthal & Morse 1986b, Baxter & Morse 1987, Wodicka & Morse 1991). Larval sensitivity to GABA analogs can be enhanced by facilitating compounds (such as exogenous lysine or certain other diamino acids), or down-regulated by prolonged larval exposure to the inducer during the pre-competent phase. Facilitating compounds are not themselves acting as inducers (Baxter & Morse 1987).

Cues That Artificially Trigger the Morphogenetic Pathway

Larval attachment and metamorphosis can be induced by substances that bind to a specific larval receptor, or by treatments that artificially trigger the larval response at some other point in the morphogenetic pathway. The latter applies to several pure chemicals that are thought to bring about larval attachment or metamorphosis by affecting elements of the signal transduction pathway such as trans-membrane ion transport, or intracellular levels of Ca^{2+} and cyclic AMP (Morse et al. 1980a, Jensen et al. 1990, Morse 1992).

An interesting example of an "artificial" cue is the potassium ion (K^+). A concentration of K^+ ~10 mM above that found in seawater triggered up to 90% of *Haliotis rufescens* larvae to attach within 72 hours (Baloun & Morse 1984, Yool et al. 1986). Metamorphosis was also induced if exposure lasted at least 20 hours (Baloun & Morse 1984). *Haliotis discus hannai* also metamorphosed in response to excess K^+ but required a higher concentration (optimally 40 mM excess) (Yang & Wu 1995). Excess K^+ induces larval attachment, but not metamorphosis, in *Haliotis iris*, *Haliotis virginea*, (Roberts & Nicholson 1997) and *Haliotis diversicolor* (Bryan & Qian 1998). This difference does not appear to result from experimental methods. The latter three species were tested with natural seawater (in contrast to the artificial seawater used with *Haliotis rufescens*) but KCl addition to natural seawater was effective for *Haliotis discus hannai* (Yang & Wu 1995) and other marine invertebrates (Pearce & Scheibling 1994). Variation of larval age, assay container volume and shape, antibiotics used, and KCl concentrations all failed to produce significant metamorphosis in *Haliotis virginea* (Roberts & Nicholson 1997).

Excess K^+ induces metamorphosis in a wide range of marine invertebrates, including several gastropod species, and it is thought to act by depolarizing excitable membranes and thereby triggering the settlement response (Baloun & Morse 1984, Pearce & Scheibling 1994). The induction by K^+ of attachment without metamorphosis in some abalone species is interesting because it suggests that there could be separate cues for attachment and metamorphosis, and differences in the signal transduction pathways for these cues.

The inducing effect of K^+ is more specific than a simple excess of cations, *Haliotis rufescens* did not settle in response to excess Na^+, Ca^{2+} or Mg^{2+} (Baloun & Morse 1984). Low external K^+ or high external Ca^{2+} inhibit induction by GABA, apparently by hyperpolarizing the receptor cells so that the ion transport brought about by the inducer does not cause cell depolarization and a nerve impulse.

These artificial cues are useful in understanding the biochemical pathways of settlement induction, and can provide useful tools for industry or research, but compounds that bind to specific larval receptors are much more likely to be ecologically relevant. These are now discussed.

Cues that Bind to Specific Larval Receptors

The inducer molecules purified from *Lithothamnium californicum* (see above) mimicked the activity of GABA in binding to GABA receptors purified from mammals (Trapido-Rosenthal & Morse 1986a). Conversely, GABA mimicked the activity of the inducer from *Lithophyllum* by binding to abalone larval receptors and triggering larval attachment and metamorphosis (Morse et al. 1979a). Several lines of evidence suggest that GABA and GABA-mimetics bind to the same receptors, and that these receptors are involved in settlement induction (Trapido-Rosenthal & Morse 1986a). The location of the receptors is unknown, but they are probably external on the larval epithelium (Barlow 1990, Morse 1990). Label bound to receptors was shed at the time of metamorphosis (Trapido-Rosenthal & Morse 1986a) but the receptors are unlikely to be on the larval velum (Barlow 1990).

Published literature contains many contradictions regarding the efficacy and effective dose of GABA (Akashige et al. 1981, Morse 1992, Table 3). The susceptibility of GABA to degradation by marine microbes (Morse et al. 1979b; Kaspar et al. 1991, Kaspar & Mountfort 1995) probably explains much of the variation in results (Searcy-Bernal & Anguiano-Beltrán 1998, Morse 1992) yet no study has quantified the decline in GABA concentrations during settlement experiments. The use of antibiotics in settlement bioassays should reduce bacterial interference (Morse et al. 1979b) but assays using recognized antibiotic treatments can still produce quantitatively variable results for the same species (e.g., see results for *Haliotis gigantea* and *Haliotis virginea* in Table 3). Such variation may result from variations in the quantity or composition of the microbial flora (Searcy-Bernal et al. 1992b) and particularly the abundance of antibiotic-resistant microbes that degrade GABA. Variability between batches of larvae may also contribute (Baloun & Morse 1984, Trapido-Rosenthal & Morse 1986b).

Another possible source of variation in GABA experiments is the composition of assay water. The concentrations of GABA required to trigger settlement can be altered orders of magnitude

TABLE 3.

Settlement response of abalone larvae to various doses of GABA.

Abalone Species	Settlement Response After (× hours)	Percent of Larvae Responding to GABA Concentrations of: 10^{-3} M	10^{-4} M	10^{-5} M	10^{-6} M	Zero	Comments	Reference
Haliotis diversicolor supertexta	Attachment (24 h)	81	77	63	60	58	10^{-3} M & 10^{-4} M GABA lethal in 72 h. No errors. Control larvae attached. Used unspecified antibiotics	Liu et al. (1986)
	Attachment (48 h)	95	96	94	91	94		
H. diversicolor	Attachment (24 h)	**48 ± 12**	**45 ± 13**	**45 ± 14**	32 ± 10[a]	22 ± 9	No antibiotics used. [a] Was significantly higher than control at 18 and 72 h	Bryan and Qian (1998)
	Shell growth (96 h)	0 ± 0	0 ± 0	**14 ± 7**	0 ± 0	2 ± 2		
H. discus hannai	Shell growth (48 h)		Toxic	**58 ± 15**	35 ± 17	0 ± 0	24 hour exposure followed by 24 hours in seawater. No antibiotics	Yang and Wu (1995)
H. discus discus	Metamorphosis (96 h)				**59 ± 7**	5 ± 0	Used 150 μg/ml penicillin + streptomycin. Data from 2 experiments.	Fukazawa et al. (in press)
	Metamorphosis (96 h)				**83 ± 10**	21 ± 11		
H. gigantea	Metamorphosis (96 h)				**82 ± 3**	7 ± 3	Used 150 μg/ml penicillin + streptomycin. Data from 2 experiments	Fukazawa et al. (in press)
	Metamorphosis (96 h)				21 ± 7	2 ± 0		
H. midae	"Settlement" (18 h)	80	80	50	50	40	No errors. Used 33 ppm penicillin.	Genade et al. (1988)
H. iris	Attachment (48 h)				**98 ± 2**	9 ± 2	Mean ± 95% CI. Used 150 μg/ml penicillin + streptomycin	Roberts and Nicholson (1997)
	Metamorphosis (96 h)				**40 ± 10**	0 ± 0		
H. virginea	Attachment (48 h)				**75 ± 5**	9 ± 3	Mean ± SE of 7 experiments. Used 150 μg/ml penicillin + streptomycin	Roberts and Nicholson (1997)
	Metamorphosis (48 h)				3 ± 2	0 ± 0		
	Attachment (48 h)	**61 ± 5**	**87 ± 1**	**73 ± 1**	4 ± 0[b]	10 ± 3	Standard error, n = 3. [b] But 2.2×10^{-6} M GABA gave 55 ± 7% attachment. Methods as in Roberts and Nicholson (1997)	R. Roberts (unpubl. data)
	Metamorphosis (48 h)	1 ± 1	1 ± 1	1 ± 0	0 ± 0	0 ± 0		
H. australis	Inferred settlement (24 h)				**73 ± 3**	48 ± 2	High settlement in controls. Survival after 7 days was 3%. Flow through system without antibiotics.	Moss (1999)
	Inferred settlement (48 h)				**85 ± 2**	60 ± 3		
	Inferred settlement (96 h)				98 ± 0.5	96 ± 2		
H. corrugata	Metamorphosis (24 h)				100 ± 0		No antibiotics used. No biofilm present	Searcy-Bernal et al. (1992a)
H. rufescens	Attachment (24 h)	96		85	96	0	Convincing data but no errors/stats given. Probably used 150 μg/ml penicillin + streptomycin (Morse et al. 1979b).	Morse et al. (1980a)
	Metamorphosis (48 h)	0		40	92	0		
	Shell growth (48 h)	0		0	88	0		
H. rufescens	Metamorphosis (24 h)				**83**	12[c]	No difference between antibiotics (100–130 μg/ml penicillin + streptomycin) (86%) and no antibiotics = 83%. No biofilm present. [c] = diatom film without GABA	Searcy-Bernal et al. (1992a)
	Metamorphosis (24 h)				**86**	12[c]		
H. rufescens	Metamorphosis (24 h)				**75 ± 10**	8 ± 8[d]	Biofilm present and no antibiotics used. [d] = biofilm without GABA	Searcy-Bernal et al. (1992b)
	Metamorphosis (24 h)				**76 ± 4**	11 ± 5[d]		
H. rufescens	Attachment (18 h)						10^{-6} M GABA did not improve settlement. Biofilms present. No antibiotics used.	Slattery (1992)

Boldface type indicates settlement significantly greater than negative controls (zero GABA column). Controls lacked any deliberate cue, except where noted. [a–d] Superscript letters refer to explanatory comments.

by dissolved substances acting on the regulatory pathway (Trapido-Rosenthal & Morse 1986b). This suggests that the use of artificial seawater might reduce variability among experiments, but caution is required in the choice of seawater. Larvae of *Haliotis discus hannai* exposed to 1 μM GABA in "ASP-M" artificial seawater (McLachlan 1964) often initiate metamorphosis more slowly than sibling larvae in natural seawater, and always fail to develop a normal post-larval shell (T. Kawamura & H. Takami,

unpubl. data). Similar results have been obtained with *Haliotis iris* where metamorphosis induced by 2 μM GABA after 5 days was ~8-fold lower in ASP-M artificial seawater (11 ± 3%, mean ± 95% CI) than in natural seawater (86 ± 3%) (R. D. Roberts unpubl. data), while larval attachment was unaffected. Interestingly, *Haliotis rufescens* does initiate metamorphosis in two artificial seawater recipes (Baloun & Morse 1984, Barlow 1990). Effects of artificial seawater on the initiation of metamorphosis may be caused by complex interactions between the ionic composition of the water and membrane depolarization of chemosensory cells (Baloun & Morse 1984).

Data on abalone larval responses to GABA are summarized in Table 3 and Figure 5. Several additional abalone species are reported to settle in response to GABA, but data have not been presented. These include *Haliotis fulgens* (Salas-Garza et al. 1994, Castro-Galvez & Searcy-Bernal 1997), *Haliotis cracherodii*, *Haliotis kamtschatkana*, *Haliotis tuberculata*, *Haliotis midae*, and *Haliotis ruber* (Morse 1984a). Most abalone species will attach within 24 hours in response to concentrations between 10^{-6} M and 10^{-3} M, but doses of 10^{-4} M and 10^{-3} M GABA are toxic within a few days (Table 3). Most abalone species initiate metamorphosis at doses of between 10^{-6} M and 10^{-5} M GABA (Table 3, Fig. 5) but higher doses inhibit shell growth (10^{-5} M) or metamorphosis and shell growth (10^{-3} M) (e.g., Morse et al. 1980a, Searcy-Bernal & Anguiano-Beltrán 1998). While high concentrations of GABA are toxic within a few days, 10–30 minute exposures to 10^{-3} M or 10^{-4} M GABA have been successfully used to induce metamorphosis in *Haliotis rufescens* (Shilling et al. 1996, Buchal et al. 1998) and *Haliotis iris* (R. D. Roberts, unpubl. data).

Haliotis virginea and *Haliotis diversicolor* larvae attach in response to GABA, but few proceed to metamorphose within four days (Table 3, Fig. 5). For *Haliotis virginea*, fine scale dose-responses (Fig. 5) and several variations of assay methodology (Roberts & Nicholson 1997) have failed to produce significant metamorphosis.

Use of GABA as a settlement cue in commercial abalone hatcheries was initially discouraged by (1) poor results from GABA in the absence of antibiotics (Morse et al. 1979b, Slattery 1992, Searcy-Bernal 1994); (2) the toxicity associated with prolonged exposure to high concentrations of GABA (Morse et al. 1980a, Akashige et al. 1981, Liu et al. 1986); and (3) the fact that GABA causes larvae to settle on horizontal surfaces (Morse et al. 1980c, Leighton 1989) where conditions unsuitable for post-larval survival can develop (Leighton 1989). However, successful large-scale induction of metamorphosis by GABA without antibiotics and in the presence of biofilms has been demonstrated (Searcy-Bernal et al. 1992b, Searcy-Bernal 1994). To help counter occasional poor results with GABA, Searcy- Bernal and Anguiano-Beltrán (1998) showed that the GABA concentration can be doubled or tripled without compromising metamorphosis or post-larval survival and growth of *Haliotis rufescens*.

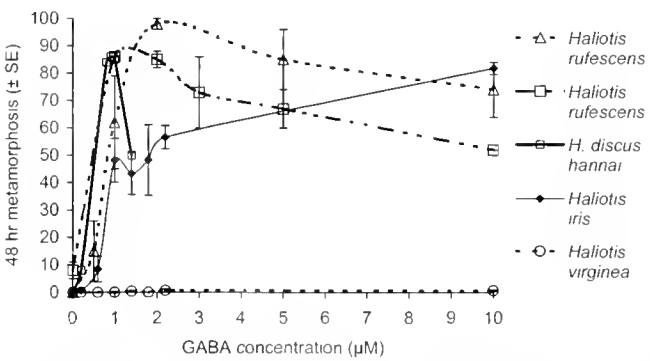

Figure 5. Metamorphosis of four abalone species in response to low doses of GABA. All assays used antibiotics and quantified metamorphosis after 48 hours of continuous exposure. The lines joining points are not fitted curves. All abalone species showed similar responses at the lowest doses with the exception of *Haliotis virginea*, which did not metamorphose at any concentration tested. Metamorphosis and shell growth of *Haliotis discus hannai* (Morse 1992, no errors presented) was lower at 1.3 μM than at 0.8 – 1.0 μM, but no other data suggest such a narrow dose response to GABA. Data for *Haliotis rufescens* (Searcy-Bernal and Anguiano-Beltrán 1998) are from two separate experiments. Methods for *Haliotis iris* and *Haliotis virginea* (R. D. Roberts unpubl. data) were as described in Roberts and Nicholson (1997).

GABA causes arrest of swimming cilia (Akashige et al. 1981, Hahn 1989c, Barlow 1990) so we expect indiscriminate settlement in the presence of GABA, but strong substrate selection in the absence of GABA (Morse et al. 1980c). In contrast, Yang and Wu (1995) found that *Haliotis discus hannai* larvae preferentially metamorphosed on certain substrates even in the presence of 1 μM GABA. These results may be explained by bacterial degradation of GABA in the absence of antibiotics.

A number of compounds that are structurally related to GABA also induced attachment of *Haliotis rufescens* larvae, presumably by binding to the larva's GABA-sensitive receptor (Morse et al. 1979a, 1980a, b). Investigation of stereochemical specificity revealed that attachment-inducing activity decreased as the carbon chain length was varied from that of GABA, and that the activity was dependent upon the presence of both the amino and carboxyl terminal groups (or on groups conferring similar functional activity) (Morse et al. 1980b). Most GABA analogs were tested only at 1 mM, while GABA was active at 1 μM (Morse et al. 1980b). Five common amino acids induced larval attachment only at concentrations three to four orders of magnitude higher than those required with GABA (Morse et al. 1980a). These studies quantified larval attachment, and did not say whether the active compounds also induced metamorphosis. *Haliotis virginea* larvae show little metamorphosis in response to GABA, yet its attachment response to several GABA-homologs (Berkett et al. 1994) mirrors that of *Haliotis rufescens* (Morse et al. 1980b), implying similar stereochemical requirements for attachment induction.

Other Chemicals whose Mode of Action is Unknown

GABA and other neurotransmitter-like molecules have been implicated in settlement induction of larval marine invertebrates other than abalone (Morse 1985). Various neurotransmitters have been tested as abalone settlement inducers but found to lack activity (Morse et al. 1980a, Akashige et al. 1981, Berkett et al. 1994, Fukazawa et al. in press). Epinephrine and serotonin stimulated the beating of the velar cilia in *Haliotis discus hannai* (Akashige et al. 1981).

Dibromomethane induces low levels of metamorphosis in *Haliotis discus hannai* (T. Seki, pers. comm.) but negligible attachment or metamorphosis in *Haliotis iris* within two days (Fig. 4). However, in both species the combination of dibromomethane and trail mucus gives rapid and complete settlement (Seki et al. 1997, Fig. 4). The biochemical basis of this synergy is not known. Mucus provides a substrate for microbial proliferation, and certain bacteria are capable of oxidizing dibromomethane (e.g., Goodwin et al.

1998). This oxidation would liberate bromide anions and (via methanol) carbon dioxide and water. *Haliotis rufescens* larvae were induced to attach by replacing 25–75% of the chloride in artificial seawater with various anions including bromide (Baloun & Morse 1984), but this represents a bromide concentration far in excess of the effective dibromomethane concentration (Fig. 4). Brominated compounds are involved in various chemical reception pathways (e.g., Wassermann et al. 1979, McKinney & Richelson 1986) but there is no evidence that such compounds are formed in the presence of dibromomethane, or are active in abalone. Organic solvents (e.g. ethanol at >0.75%) can induce abalone larval settlement (Fenteany & Morse 1993). However, the methanol generated by oxidation of dibromomethane would be short-lived, so would not reach concentrations expected to induce settlement in abalone larvae.

Thyroxine (T_4) at 33–132 μM induced 85% metamorphosis of *Haliotis rufescens* larvae in 44 hours (Carpizo-Ituarte & Rosa-Velez 1993). The thyroid hormones T_4, 3, 5, 3′-triiodothyronine (T_3), 3, 3′, 5′-triiodothyronine (rT_3) and tetraiodothyroacetic acid (tetrac) induced metamorphosis of *Haliotis discus discus*, *Haliotis gigantea* and *Haliotis iris* at concentrations as low as 0.1–1 μM, while 3, 5-diiodothyronine (3, 5-T2) and diiodotyrosine (DIT) were inactive (Fukazawa et al. in press, and unpubl. data). Thyroid hormones are involved in the metamorphosis, development or metabolism of vertebrates and some invertebrates (e.g., Galton 1992, Eales 1997, Johnson 1997). The abalone data support the case for an ancient evolution of the regulatory functions of thyroid hormones (Johnson 1997).

Algal extracts are often toxic to abalone larvae and can inhibit normal larval attachment, metamorphosis or shell growth (Morse & Morse 1984a, Roberts & Nicholson 1997). In the case of the brown alga *Dilophus okamurai* and *Haliotis discus hannai* the toxic compounds were found to be two spatane-type diterpene alcohols (Taniguchi et al. 1989, Shiraishi et al. 1990). Various environmental contaminants and temperature can also interfere with normal abalone larval settlement (Morse et al. 1979b, Steinbeck 1980, Conroy et al. 1996, Raimondi et al. 1997). Certain antibiotics have also caused positive or negative interference in abalone settlement assays (see above).

DISCUSSION

Do Attachment and Metamorphosis Occur Spontaneously?

The normal behavior of competent abalone larvae includes periods of crawling and substrate testing (Seki & Kan-no 1981a). Thus, it is not surprising that even clean, young negative controls with antibiotics often have a small proportion of larvae attached (e.g., Roberts & Nicholson 1997). This background level of attachment probably occurs in the absence of any cue but is quite distinct from spontaneous metamorphosis.

It is common to record a very low percentage of metamorphosis in negative controls, even in the presence of antibiotics (Table 3). Varying levels of apparently spontaneous metamorphosis do occur in aging abalone (Roberts & Lapworth 2001, Takami et al. unpubl. data, Searcy-Bernal pers. comm.). Given that microbes can induce metamorphosis, it is difficult to be confident that any metamorphosis occurred spontaneously unless the larval cultures are axenic.

Abalone larvae become increasingly sensitive to metamorphic cues during the week or so after first attaining competence (Morse 1984b, Barlow 1990, Moss & Tong 1992a) probably because of continued synthesis of chemical receptors by the aging larva (Degnan & Morse 1995). This will serve to heighten the larva's sensitivity to sub-optimal cues and may increase the chance of metamorphosis occurring without any obvious trigger. A more critical period for abalone comes later, when larvae are nearing the end of their life expectancy and are faced with the choice between spontaneous metamorphosis or death. There is no information on changes in settlement-related biochemical pathways in abalone larvae during this period. In some animals metamorphosis is regulated by internal inhibitory compounds as well as external cues (Youson 1997). If such a mechanism existed in abalone, the degradation of the inhibiting compound in aging larvae would offer a possible explanation for spontaneous metamorphosis.

Timing and Endpoint of the Settlement Response—Are There Multiple Cues?

The timing and end point of the abalone settlement response varies greatly among cues. Attachment can be uncoupled from metamorphosis in at least several abalone species (Leighton 1972, Kawamura & Kikuchi 1992, Roberts & Nicholson 1997, Bryan & Qian 1998, Moss 1999). A wide array of chemicals and organisms induce attachment of abalone larvae within a day, but few give rapid metamorphosis. It is possible that larval attachment and metamorphosis can be triggered by separate cues (Roberts & Nicholson 1997, Seki et al. 1997, Bryan & Qian 1998) acting via independent or interacting biochemical pathways.

The slow increase in percent metamorphosis commonly seen among larvae incubated with bacteria (Morse et al. 1979b) and many diatom strains (e.g., Kawamura & Kikuchi 1992) could be explained by infrequent encounters between larvae and sufficient quantities of inducers. The cues may be continually produced and degraded by microbes (Morse et al. 1979b, Kaspar et al. 1991, Searcy-Bernal et al. 1992b, Kaspar & Mountfort 1995) but not present in sufficient quantity to induce a synchronized settlement response.

An interesting aspect of induction by K^+ is that metamorphosis induction only occurred if the treatment was applied for at least 20 hours (Baloun & Morse 1984). In the case of coralline algae, or other fast-acting cues, metamorphosis begins within several hours (e.g., Barlow 1990, Seki 1997). Such differences (if not caused by confounding effects such as larval age) may provide clues about the biochemical pathways of settlement induction.

The GABA-mimetic receptor is strict in its stereochemical specificity (Morse et al. 1980b). Many of the molecules that induce abalone attachment or metamorphosis probably trigger the morphogenetic pathway downstream of the GABA-mimetic receptor (Morse 1992). The activity of other molecules, such as dibromomethane and thyroid hormones, cannot be readily explained by the existing model (A. Morse 1991, D. Morse 1992) and probably act at larval receptors not yet characterized. The diverse array of natural substances capable of inducing some degree of abalone larval attachment or metamorphosis suggests that either (1) there are multiple cues/receptors that can trigger settlement, or (2) the cues are widely available in marine biota.

Synergistic Effects Between Cues

The best described example of synergy between abalone larval settlement cues is the interaction between the regulatory and morphogenetic pathways proposed for *Haliotis rufescens* (Trapido-Rosenthal & Morse 1985, Trapido-Rosenthal & Morse 1986a, Trapido-Rosenthal & Morse 1986b, Morse 1991). Diamino acids

can amplify larval sensitivity to GABA-mimetic molecules by up to 100 fold. Other examples of synergy involve GABA plus coralline algal extract, GABA plus diatom film (Roberts & Nicholson 1997) and dibromomethane plus mucus (Seki et al. 1997, Fig. 4). The biochemical basis of these latter synergies is not known. Both cases involving GABA have a crude organic matrix (diatom film or crude extract) as the second cue. This matrix could yield diamino acids that act through the regulatory pathway. Alternatively, the synergy may involve inducers/receptors not yet characterized.

Settlement Cues for use in Hatcheries

Unpredictable settlement rates complicate post-larval culture by giving variable post-larval densities. Low or intermediate rates of settlement are very unpredictable, so hatcheries should aim for consistently high percentage metamorphosis. Ideally metamorphosis would also occur very rapidly so that full water flow and full aeration can be resumed as soon as possible, without risking loss of larvae. Very few settlement cues are able to deliver on these requirements.

Crustose coralline algae meet these settlement requirements but are generally regarded as unsuitable for use in hatcheries because they may harbor predators, are slow growing, must remain wet and lack established culture methods. Their potential use has not been fully evaluated, and selected species may prove valuable with further research. Very thin crusts grow relatively quickly (McShane 1996) and do not host the predatory worms that live within thicker crusts (Morse et al. 1979b, Naylor & McShane 1997). The food value of corallines for post-larval abalone is generally low (Leighton 1989, Kitting & Morse 1997), so they would need to be supplemented with diatom food. Some corallines will inhibit the growth of diatoms (A. Krsinich, pers. comm.) but corallines need only cover a small proportion of plates to trigger settlement. Controlled culture of certain corallines is feasible (S. Daume, pers. comm.) and growth rates are not critical if only a small proportion of the surface needs to be covered at settlement.

GABA is a very convenient and simple cue, and is used in some commercial hatcheries, particularly in Mexico and the USA (Morse 1992, Searcy-Bernal 1994, Searcy-Bernal & Anguiano-Beltrán 1998). Its performance appears to vary among abalone species, and between occasions (e.g., Table 3). GABA is susceptible to degradation by microbes but this can be counter-acted to some extent by doubling the GABA concentration (Searcy-Bernal & Anguiano-Beltrán 1998) or by settling larvae in clean tanks and adding diatom food a few days later (Searcy-Bernal et al. 1992a; Roberts et al. 2001). It may also become a non-issue if larval sensitivity could be affordably enhanced 10-fold by addition of diamino acids (Trapido-Rosenthal & Morse 1985), or if short exposures to high concentrations of GABA (e.g., Shilling et al. 1996, Buchal et al. 1998) prove practical in hatcheries. GABA causes arrest of swimming activity, so larvae attach and metamorphose largely on non-vertical surfaces. This is impractical in many systems (e.g., Leighton 1989), including those employing stacks of vertical plates (although see Salas-Garza et al. 1994). Angled plates may catch a large proportion of larvae, but a more elegant alternative would be to utilize a combination of diatoms and GABA. A diatom film is likely to induce attachment over filmed plates or tank surfaces within a day or so. Once attachment had occurred, addition of GABA would trigger metamorphosis of attached larvae, provided the GABA had time to act before it was degraded by microbes.

Ungrazed diatom films alone provide little certainty for larval settlement in hatcheries. Few strains induce consistently high metamorphosis, and the need for a dense film at settlement can rapidly lead to excessive biofilm and adverse conditions for post-larvae. By contrast, pre-grazed films generally induce high rates of attachment and metamorphosis, and the reduction of biomass by grazing will help avoid excessive biofilm. Films grazed for several weeks are expected to be more reliable because of the selection for certain algae that are favorable for settlement. However, even young films grazed for one to three days appear to be efficient settlement inducers. GABA could be used in conjunction with diatom films, as discussed above.

Differences and Similarities Among Abalone Species

Comparison between species is often complicated by differences in methodology (see above). A few studies have compared abalone species using equivalent assay methods, and provide compelling evidence of real differences in settlement responses between abalone species. Two examples (reviewed above) are the response of *Haliotis iris* and *Haliotis virginea* to GABA (Table 3, Fig. 5) and the response of *Haliotis rubra* and *Haliotis laevigata* to several diatom strains (Daume et al. 1999b). These examples warn of differences in chemical signaling that result in fundamentally different responses between abalone species. However, in many respects the larval settlement response appears to be conserved across abalone species. Examples reviewed above include the widely observed positive response to crustose coralline algae and pre-grazed diatom films.

Ecological Relevance of Chemical Inducers

Chemical extraction procedures liberate hundreds of compounds from the source material without preserving the spatial or stereochemical context of the intact surface. This creates the potential to identify inducers that are merely artifacts of the extraction and purification process (e.g., Morse 1990). It is accordingly difficult to prove that a compound isolated from a settlement substratum is ecologically relevant. The extensive work by Daniel Morse and colleagues, working with *Haliotis rufescens*, presents a compelling case for a GABA-mimetic peptide from *Lithothamnium californicum*. However, this molecule could be just one of many inducers available in the marine environment. Dibromomethane has also recently been suggested as an ecologically relevant settlement inducer for abalone (Seki et al. 1997), but further evidence is required to demonstrate this. Most other pure chemicals that induce abalone settlement are not proposed to be ecologically important.

Is Settlement Exclusive to Coralline Algae in the Wild?

This review has noted the close association between juvenile abalone and crustose coralline algae, and the consistently excellent inducing activity of corallines in laboratory settlement experiments. However, experiments show that abalone larvae can also settle in response to many substrata that are present in abalone habitat including other macroalgae, abalone trail mucus, and biofilms (reviewed above). These experiments are artificial in that they usually confine larvae with a substrate. In the wild, larvae may be more likely to settle on first contact if they land on coralline algae rather than some less effective cue. This contention is supported by the rapidity with which abalone metamorphose on coralline algae compared to many other cues in laboratory assays. Settlement choice experiments in laboratory conditions would be

instructive, and have already demonstrated that larvae exhibit preferences for certain coralline algal species (Daume et al. 1999a).

Artificial collectors conditioned with algal films or pre-grazed films then placed in abalone habitat do contain abalone post-larvae after 1–8 weeks in the sea (Keesing et al. 1995, Nash et al. 1995). These data suggest that abalone larvae do naturally settle on surfaces other than corallines, but it is possible that corallines had colonized the plates during conditioning or deployment and triggered larval settlement. Settlement deterrents from algae (e.g., Taniguchi et al. 1989) and sessile invertebrates may play a major role in discouraging settlement on many surfaces, although Morse and Morse (1984a) rejected the idea that abalone's preference for corallines was due to the toxicity of other algae tested.

The distribution of post-larvae or juveniles on natural surfaces can be influenced by post-settlement survival or migration, so it is not necessarily a reliable indicator of settlement sites. Corallines do not provide a perfect surface for abalone post-larvae. Corallines appear to give relatively slow growth rates of post-larval abalone when compared to benthic diatoms (Kitting & Morse 1997, Takami et al. 1997, Kawamura et al. 1998), they can harbor predators (Morse et al. 1979b, Shepherd & Daume 1996, Naylor & McShane 1997) and they generate moderately steep gradients in boundary layer water chemistry (Kaspar 1992). However, they may still represent a better option than other surfaces.

Research Needs

The ability of this review to generalize about the effectiveness of abalone settlement cues has been hampered by differences in methodology between experiments, and often by the lack of data on metamorphosis as distinct from larval attachment. It is hoped that this review will encourage the use of appropriate terminology and methodology. Most abalone larval settlement experiments will aim to either: (1) separate treatment effects from all possible alternatives in tightly controlled laboratory conditions; (2) determine the usefulness of selected treatments to commercial abalone farming; or (3) attempt to describe settlement patterns or processes in the sea. Small-scale experiments attempting to mimic hatchery or field conditions risk falling between two aims. Laboratory experiments will continue to be necessary and informative in research on abalone settlement cues, but there is currently a disproportionate amount of laboratory data and a corresponding need for laboratory results to be assessed in commercial abalone farming systems or natural habitats.

Despite many years of commercial abalone farming around the world there are very few published data describing settlement success in full-scale commercial conditions. The rigor of laboratory studies needs to be coupled with the reality of commercial scale hatchery conditions, and quantitative, microscopic observations made over time. This will create challenges in terms of controlling experimental conditions and obtaining adequate experimental designs and sub-samples, but when achieved will yield very valuable information. Further research on the hatchery application of coralline algae, trail mucus and commercially available chemicals such as GABA would be valuable. A chemical inducer that could be bound to plastic surfaces would be ideal for the many hatcheries that use vertical plates. Hatchery experiments should aim to assess cost-effectiveness as well as biological success. Issues such as practicality and predictability will influence the cost-benefit equation.

The chemistry of abalone settlement induction remains very poorly known, with the exception of the GABA-mimetic system studied in *Haliotis rufescens*. It would be interesting to screen for proposed "natural" cues (i.e., GABA-mimetics and dibromomethane) on various inductive substrates, to determine whether known cues explain the activity of diverse substrates, or whether there are multiple alternative pathways for settlement induction in abalone. The chemical basis of settlement induction by biofilms should be a priority, as biofilms induce settlement in a very wide range of invertebrates, and have economic importance in abalone farming and biofouling control. The mechanisms underlying the synergy between cues are academically interesting and have potential to aid abalone farming.

The role of bacteria in settlement induction by diatom films and trail mucus is unknown. Bacteria will certainly be degraders of organic inducer molecules, but may also play a critical role as producers or modifiers of inducers. Settlement assays conducted under axenic conditions would be useful in separating microbial effects from other cues, and in determining whether abalone can metamorphose spontaneously.

Field experiments offering a range of natural substrata should help clarify the apparent paradox between the wide range of potential inducers for abalone settlement and the tight association between juvenile abalone and crustose corallines in the wild (McShane 1995). The extent of settlement deterrence by algae and sessile invertebrates (McShane 1995), and further examination of preferences for certain coralline species (McShane 1996), could be incorporated in these studies. Observations from larval settlement through the post-larval period would provide direct evidence about the relative contribution of settlement and post-settlement processes in determining spatial variation in juvenile recruitment between microhabitats. These studies would be useful in site selection for larval reseeding programs.

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EFFECT OF BIOFILM DENSITY ON GRAZING AND GROWTH RATES OF *HALIOTIS FULGENS* POSTLARVAE

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ABSTRACT Grazing rates of *Haliotis fulgens* postlarvae of different ages (7, 15, 30, 45 and 60 days), feeding on the cultured diatom *Navicula incerta*, were estimated in 10 ml sterile plastic dishes previously inoculated with different densities of the diatom (100–4,000 cells/mm^2). Postlarvae (3–7 per dish) were allowed to graze for two to three hours and video recordings were taken to estimate postlarval size and grazing rates by digital image analysis. Seawater was changed every other day and postlarvae were measured again after six to eight days to estimate growth. Grazing and growth rates of postlarvae older than 15 days increased linearly with biofilm density. The highest grazing rates for seven and 60 day-old abalone were 79 and 10,999 cells/postlarva/hour, respectively. The most important increase in grazing activity occurred between ages 45 and 60 days when postlarvae reached 1.5–2.0 mm and started the formation of the first respiratory pore. Implications for the management of production systems are discussed.

KEY WORDS: abalone postlarvae, *Haliotis fulgens*, grazing rates, biofilms

INTRODUCTION

In culture systems, abalone (*Haliotis spp.*) postlarvae feed on biofilms dominated by benthic diatoms and bacteria (Hahn 1989, Kawamura 1996). A key problem in this production stage is how to keep an adequate balance between postlarval feeding and diatom supply. If grazing is excessive, starving conditions may severely impact postlarval survival and growth. In the opposite situation, overdeveloped biofilms may also create deleterious conditions leading to massive losses of postlarvae (Ebert & Houk 1984, Hahn 1989, Searcy-Bernal et al. 1992, Tong & Moss 1992, Searcy-Bernal 1996).

Most studies on abalone postlarval feeding, have focused on the effect of different diatoms on postlarval growth, on the factors affecting their food value (Kawamura et al. 1998a, Roberts et al. 1999), and on changes in diatom selectivity and digestion as postlarvae grow (Norman-Boudreau et al. 1986, Matthews & Cook 1995, Seki & Taniguchi 1996, Kawamura et al. 1998b, Takami et al. 1998, Roberts et al. 1999).

The quantitative assessment of postlarval grazing is crucial to understand these complex culture systems and to suggest better management options. However, despite its importance, this issue has received little attention. Martínez-Ponce and Searcy-Bernal (1998) provided the first estimates of abalone postlarval grazing, by measuring cleared areas by *H. rufescens* postlarvae fed known densities of the benthic diatom *Navicula incerta*, using the digital analysis of video-recorded images. Roberts et al. (1999) estimated consumption rates of *H. iris* postlarvae fed different diatom strains, by determining fecal production. Both studies concluded that grazing increases rapidly as postlarvae grow, after the first one to two weeks after settlement. Body size is one of the main factors determining grazing rates of juvenile abalone (Marsden & Williams 1996).

Preliminary data suggest that, besides postlarval size and diatom species, the density of biofilm may also affect postlarval grazing and growth rates. This article explores this relationship for *Haliotis fulgens* (Philippi 1854) fed the diatom *N. incerta*. According to general concepts on the response of consumers to food abundance (Valiela 1995), grazing rates were expected to increase with diatom density until saturation values were reached (type II functional response).

MATERIALS AND METHODS

Veliger larvae were produced at the hatchery of Cooperativa Emancipación, S.C.L. (Puerto Nuevo, B.C.S., México) and transported to the Instituto de Investigaciones Oceanológicas (I.I.O.) (Ensenada, B.C.), where they were reared in 1-μm filtered, UV treated seawater with chloramphenicol (10mg/l) added, changed every day. Competent larvae were induced to metamorphose in 20L plastic containers with 1.5 μM gamma-aminobutyric acid (Searcy-Bernal and Anguiano-Beltrán, 1998). The cultured diatom *Navicula incerta* (~200 cells/mm^2) was added at day 2 after settlement. This benthic diatom (~13 μm length) was isolated from local coastal waters and is currently used in research and commercial operations in México. Postlarvae were cultured in these containers with seawater (treated as described) changed every two days for the first two weeks, and with flowing 5-μm filtered seawater (~300 ml/min) thereafter. Constant fluorescent illumination (~50μE/m^2/s) and gentle aeration were provided. Temperature fluctuated between 17 and 19°C.

Postlarvae were collected from the culture system at days 7, 15, 30, 45 and 60 after settlement and introduced to containers (10-ml wells of sterile culture dishes, 3–7 postlarvae per well), previously inoculated with *N. incerta* at target densities of 100, 500, 750, 1000, 2000, and 4000 cells/mm^2 (with the exception of trials with seven-day-old postlarvae where the higher density was substituted by 1,500 cells/mm^2). Before the preparation of experimental densities, batch cultures of this diatom were immersed in an ultrasound bath for 3–5 min and filtered through a 20 μm sieve to disperse cells. A randomized block design with three replicates per diatom density was originally considered for each postlarval age (with the exception of age 45 days where only two replicates were used).

Two to three hours after the introduction of postlarvae to the experimental units, video-images were recorded (Sony SSC-C374 high resolution camera) on an inverted microscope (Meiji Techno) and digitally analyzed (NIH Image 1.59, PowerPC Macintosh computer) to measure postlarval shell length, and to determine

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cleared areas and actual diatom densities. Grazing rates (cells/postlarva/h) were estimated by multiplying cleared areas by diatom densities. Previous experience had shown that postlarvae start feeding activity almost immediately after their introduction in vessels (i.e. in less than 2 min) and that *N. incerta* growth and recolonization of cleared areas are negligible during this 2–3h period (Martínez-Ponce & Searcy-Bernal 1998).

Experimental containers were maintained for six days (19 ± 1°C, 4 μE/m^2/s) with seawater (treated as described) changed every 48 hours (with the exception of 7-day-old postlarvae). Postlarval shell length (SL) was measured at the end of this period to determine growth rates (μm/d).

Because of the high variability in diatom density among replicates (despite the efforts to minimize it), data (average growth and grazing rates) were analyzed considering wells individually. To estimate the maximum grazing and growth rates for each postlarval age, the three highest values were averaged.

RESULTS

Figure 1 shows the shell length of *H. fulgens* postlarvae collected from the culture system at the different ages tested. In the first 15 days growth was slower (14.9 μm/d) than in the period from days 15 to 60 (33.4 μm/d). Mortality of postlarvae of all ages in wells was negligible at all diatom densities during the experimental period (<5%).

Grazing rates of seven-day-old postlarvae averaged 54.5 cells/postlarva/h and were independent from diatom density ($r = 0.319$, $P > 0.05$) (Fig. 2). However, at age 15 days grazing rate increased dramatically, averaging 362 cells/pl/h and showing a positive and significant linear association with diatom density ($r = 0.773$, $P < 0.01$) (Fig. 3a) which was not significant for postlarval growth rates ($r = 0.345$, $P > 0.05$) (Fig. 3b).

At postlarval ages of 30, 45 and 60 days, the dependence of grazing and growth rates on diatom density was stronger (Figs. 4, 5, 6 respectively). Within the range of densities tested, most of these relationships were adequately described by linear regressions, although for 30-day-old postlarvae an asymptotic trend for grazing at higher diatom densities was observed (Fig. 4a). A logarithmic fit to these data resulted in an $r = 0.829$ which is higher than the value for the linear model ($r = 0.655$). In addition, at this age, linear correlation between growth rate and diatom density was not significant because of an outlier (slow growth at the highest density).

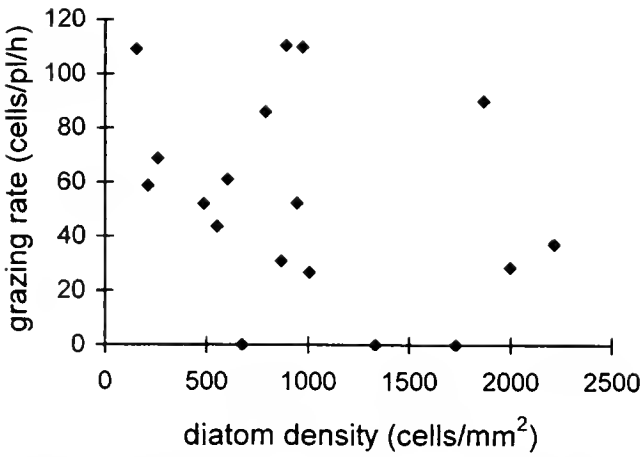
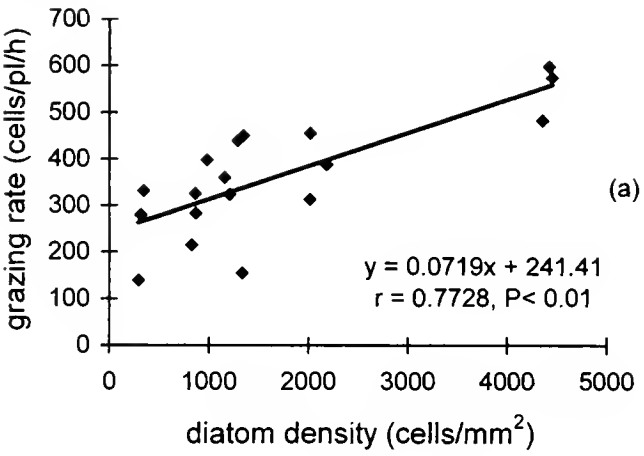


Figure 2. Grazing rates of seven-day-old postlarvae of *H. fulgens* at different densities of the diatom *Navicula incerta*. Data are means for 5–7 postlarvae in experimental units.



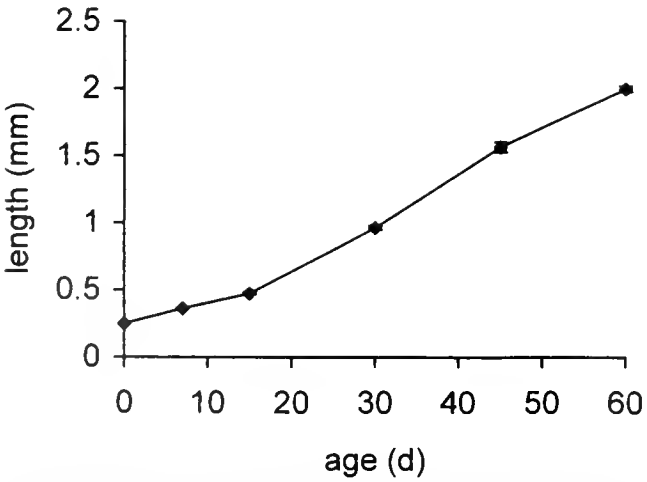


Figure 1. Shell length (mm) of *H fulgens postlarvae* collected from the culture system at different ages (days after settlement) Data are averages of 36–90 postlarvae.

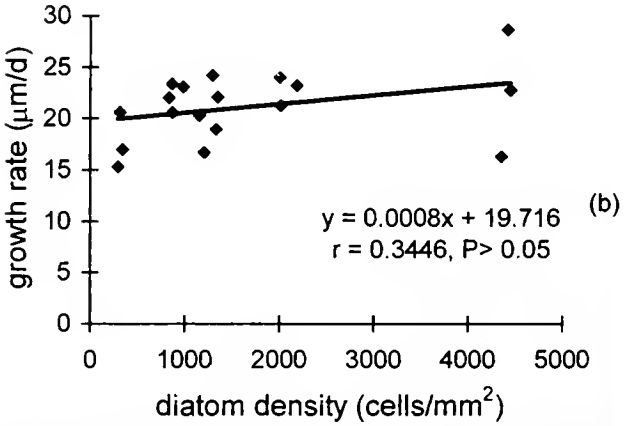
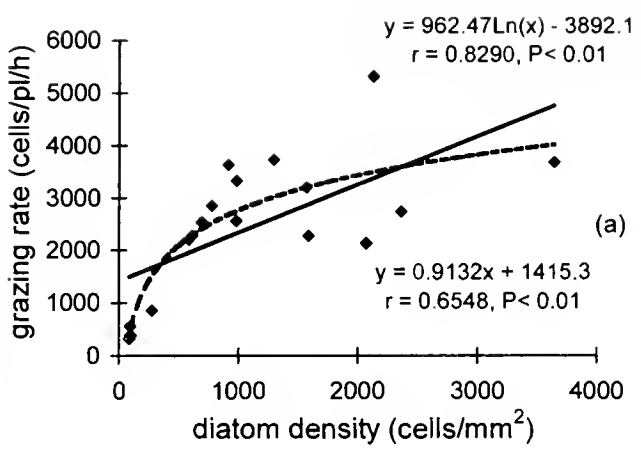
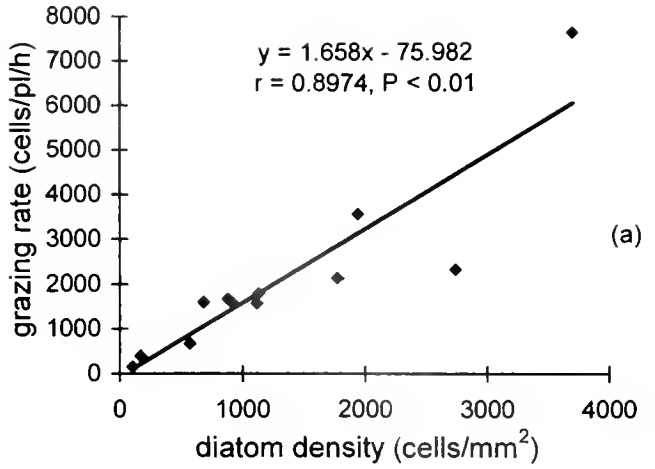


Figure 3. Grazing (a) and growth (b) rates of 15-day-old postlarvae of *H. fulgens* at different densities of the diatom *Navicula incerta*. Data are means for 5–7 postlarvae in experimental units.





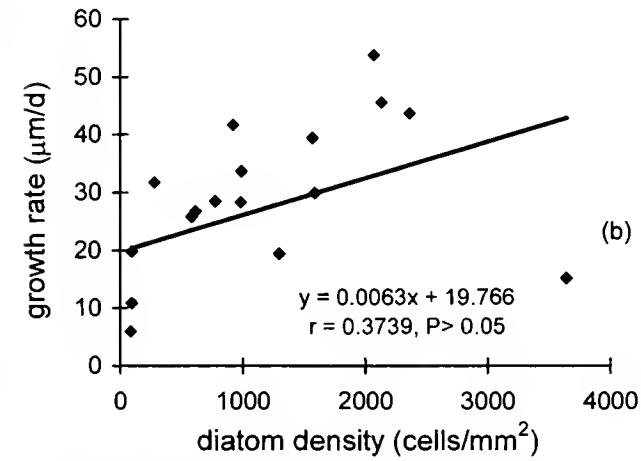


Figure 4. Grazing (a) and growth (b) rates of 30-day-old postlarvae of H. fulgens at different densities of the diatom *Navicula incerta*. Data are means of 3–5 postlarvae in experimental units.

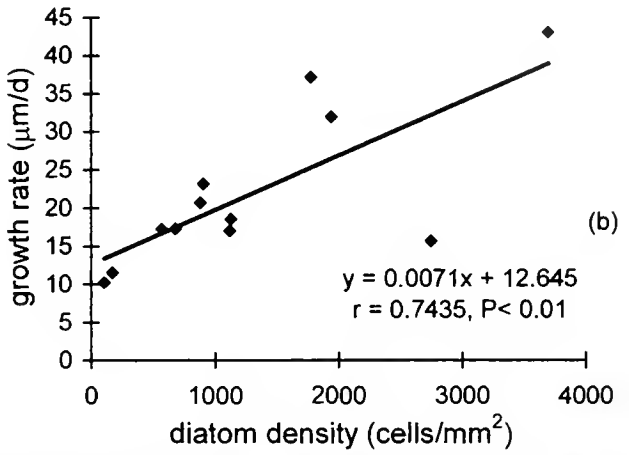


Figure 5. Grazing (a) and growth (b) rates of 45-day-old postlarvae of *H. fulgens* at different densities of the diatom *Navicula incerta*. Data are means of 3–5 postlarvae in experimental units.

The slopes of linear regressions of grazing rates on diatom density increased with postlarval age, but this trend was not observed for postlarval growth rates (Figs. 2–6).

Estimates of maximum grazing and growth rates are presented in Table 1. Maximum grazing rates ranged between 79 and 10,999 cells/pl/h for postlarval ages of seven days and 60 days, respectively, and increased non-linearly with postlarval shell length, following a trend consistent with a power function (Fig. 7). Maximum growth rates did not follow a similar pattern, reaching values of 25.6, 47.6, 37.4 and 39.6 µm/d for ages at 15, 30, 45 and 60 days, respectively.

DISCUSSION

Effects of Biofilm Density on Postlarval Grazing Rates

This study shows that grazing rates of *H. fulgens* postlarvae increase abruptly and become strongly dependent on diatom density the first one to two weeks after settlement. Grazing by earlier postlarvae (seven days) was minimal and independent of food abundance, in agreement with observations on postlarvae of this and other abalone species (Matthews & Cook 1995, Kitting & Morse 1997, Kawamura et al. 1998b, Martínez-Ponce & Searcy-Bernal 1998, Roberts et al. 1999).

Only for 30-day-old postlarvae, results are consistent with a type II functional response of grazing rates to diatom density (Fig. 4a). Grazing rates level off at about 3,500 cells/pl/h, corresponding to an approximate diatom density of 2,000 cells/mm^2. This type of functional response is common in marine consumers (Valiela 1995) and has been recently documented for juveniles (3–4 cm SL) of the abalone *H. asinina* feeding on macroalgae (Tahil & Juinio-Menez 1999) and for the periwinkle *Littorina littorea* feeding on biofilms (Sommer 1999).

In a previous study with one-month-old *H. rufescens* postlarvae an asymptotic trend in grazing rates was also detected, leveling off at around 1,000 cells/pl/h when *N. incerta* reached densities of ca. 1,000 cells/mm^2 (Searcy-Bernal & Cook, in prep.). Differences with *H. fulgens* might be explained by the smaller size of *H. rufescens* postlarvae (~750 µM SL), the lower temperature (~16°C) or might reflect actual inter-specific differences. In high diatom densities (~>2,000 cells/mm^2) many of the *H. rufescens* postlarvae crawled to the walls of wells almost immediately after their introduction into the bottom of containers. This behavior was never observed in *H. fulgens* regardless of the postlarval age or diatom density tested, which suggests that this species is able to cope with denser biofilms.

At the other ages tested—except at age seven days—grazing rates by *H. fulgens* postlarvae increased linearly with biofilm den-

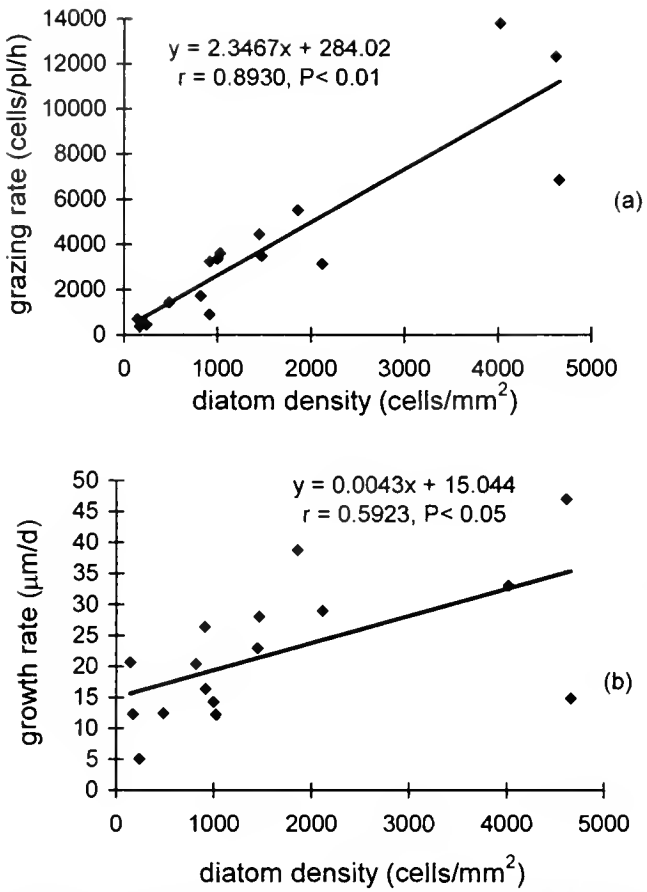


Figure 6. Grazing (a) and growth (b) rates of 60-day-old postlarvae of *H. fulgens* at different densities of the diatom *Navicula incerta*. Data are means for 3–5 postlarvae in experimental units.

sity and no evidence of leveling-off was observed. For older postlarvae (45- and 60-day-old), this pattern may indicate that densities tested were not high enough to produce an asymptotic trend in grazing activity. However, grazing rates of 15-day-old postlarvae would have been expected to level off at a diatom density lower than the correspondent for 30-day-old abalones. This result may be partially influenced by the low grazing activity by 15-day-old postlarvae, which increased dramatically (~10-fold) at age 30 days old (Fig. 3a and Fig. 4a).

TABLE 1.

Maximum grazing and growth rates of *H. fulgens* postlarvae of different ages. Data are means of the three highest values and standard errors are shown in parenthesis.

Age (d)	Length (µm)	Max. Grazing Rate (cells/pl/h)	Max. Growth Rate (µm/d)
7	363.4	78.9	—
	(2.1)	(15.4)	
15	473.5	553.5	25.6
	(8.0)	(35.6)	(1.5)
30	963.1	3,903.1	47.6
	(16.1)	(752.9)	(3.1)
45	1,563.7	4,984.5	37.4
	(37.6)	(2,646.7)	(3.9)
60	2,000.7	10,998.7	39.6
	(22.9)	(2,110.9)	(3.3)

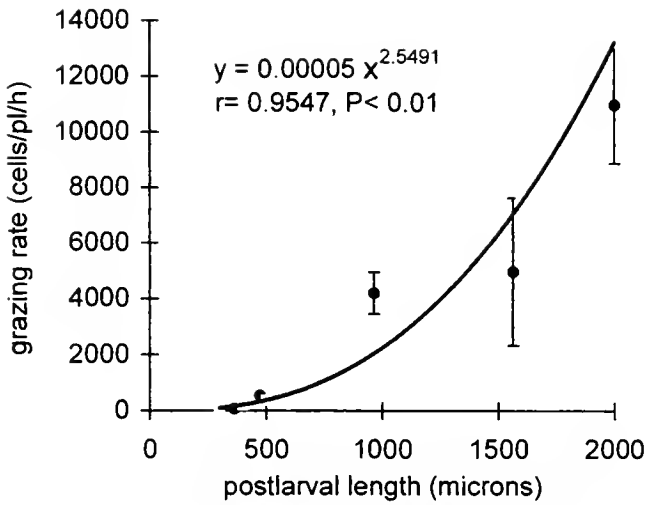


Figure 7. Relationship between maximum grazing rate and length in postlarvae of *H. fulgens*.

Effects of Biofilm Density on Postlarval Growth Rates

In general, postlarval growth rates also increased linearly with diatom density, following similar trends than grazing rates (Figs. 3–6). This may reflect the obvious relationship between food consumption and growth, despite the fact that grazing rates correspond to the first two to three hours of the experiment and growth rates to the total six-day period. Since diatom density at the end of that period was usually lower than the initial (although probably proportional), the quantitative relationships between growth and biofilm density can only be considered as a first approximation.

It should be noted, however, that moderate differences of diatom densities may produce dramatic differences in growth. In trials with 30, 45, and 60 days postlarvae, growth rates after 6 days at 2,000 cells/mm^2 were more than 50% higher than those at 1,000 cells/mm^2 (Figs. 4b–6b). This short-term effect, in conjunction with patchiness of postlarvae and biofilm densities (and composition) in culture systems, may create local areas of differential growth, influencing the size heterogeneity commonly observed since early culture stages (Hahn 1989). This initial size variability would be further maintained and even increased by intra-specific competition. Therefore, mechanisms underlying growth heterogeneity in abalone postlarvae, may not be only genetic or related to metamorphosis induction as commonly suggested (Hahn 1989, Morse 1992), but may also relate to early feeding history.

The effect of diatom density on abalone postlarval growth, should also be considered for research purposes. For instance, a study may conclude that postlarvae grow better in diatom A than in diatom B, only because diatom A was provided at a more adequate density.

Grazing and Growth Rates of Postlarvae as a Function of Shell Length (SL)

According to the power function fitted, (Fig. 7) maximum grazing rates were proportional to $SL^{2.55}$, which indicates a closer relationship to postlarval volume or biomass. Since, as discussed above, the maximum grazing rates calculated here probably underestimate asymptotic values for postlarval ages of 45 and 60

days the actual coefficient of that equation was probably higher. Roberts et al. (1999) found that fecal production (as an indicator of ingestion rate) of *H. iris* postlarvae was proportional to $SL^{2.73}$, which is consistent with our results.

Estimates of maximum growth rates were not proportional to postlarval age or length. The lowest value (25.6 μm/d), corresponded to 15-day postlarvae and the highest (47.6 μm/d) to 30-day postlarvae, reflecting an increased effect of diatom grazing. Again, potential growth rates for 45-day and 60-day postlarvae were probably underestimated as a consequence of the range of diatom densities tested. The low variability in growth rate of postlarvae during development, is consistent with the nearly linear increase in SL after the first one to two weeks of abalone postlarval culture, reported here (Fig. 1) and in other studies (Searcy-Bernal et al. 1988, Flores-Aguilar 1989, Kawamura et al. 1998b, Martínez-Ponce & Searcy-Bernal 1998).

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LARVAL DEVELOPMENT IN *HALIOTIS ASININA* LINNAEUS

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ABSTRACT Larval development of *Haliotis asinina* Linnaeus was observed under water temperatures of 25 C, 28 C, 31 C, and 34 C from fertilization to the formation of the fourth tubules on the cephalic tentacles. The larvae had 42 stages of development. The time-period of larval development depended on the water temperature: it was 65, 49, 41 and 41 h at water temperatures of 25 C, 28 C, 31 C, and 34 C, respectively. After settlement, the development of post-larvae through the formation of the first respiratory pore was observed under room temperatures (28°C–35°C). The velum was shed and the mantle began to secrete a new shell. Mouth, radula and digestive organs were developed by the third day of settlement. The heart was seen on the fourth day. The prominent structure on the roof of the mantle cavity was the etenidium. The second pair of epipodial tentacles began to form and the eyestalks were completely developed on the eighth day after settlement. From day 9 to day 24, the post-larvae increased in shell size and number of epipodia and tubules on the cephalic tentacles. The etenidium became more developed. The first respiratory pore began to form on day 24 to 30.

KEY WORDS: abalone, larval development, *Haliotis asinina*

INTRODUCTION

The life cycle of abalone is composed of larva, post-larva, juvenile and adult stages. The larval stage begins with fertilization and ends with the formation of the fourth tubule on the cephalic tentacles (Hahn 1989a). Then, the larvae begin to settle and undergo metamorphosis. The deposition of the peristomal shell marks the transition from the larval to the post-larval stage (Hahn 1989a). The post-larval period continues until the formation of the first respiratory pore (notch stage). From the notch stage until sexual maturity, the abalone is called a juvenile (Leighton 1974).

Larval development is a gradual process that does not occur in discrete stepwise stages. However, various stages can be recognized during larval and post-larval development (Hahn 1989a). In *Haliotis discus hannai* (Ino 1952), there are 41 distinct larval stages with recognizable external features, from fertilization until initiation of metamorphosis (Seki & Kan-no 1977). These morphological features and the order of their appearance in larval developmental stages provide guidelines for studies of larval development in other abalone species. After the larvae settle, 11 recognizable external features appear following the appearance of the cilia in the mantle cavity. These are confirmed to be the characteristics occurring before metamorphosis (Seki 1997).

The larval development rate is measured by the time required for larvae to exhibit features distinctive to each stage (Hahn 1989a). Water temperature is the major factor that affects the larval development rate (Leighton 1974, Ebert & Houk 1984, Owen et al. 1984, Hahn 1989c). Abalone seed production in Thailand has remained in a primitive and small-scale stage for a long period. This is partly because of the difficulty in planning spawning, and the high mortality in the early developmental stage (Seki 1997). Studies of developmental morphology, behavior and the biological requirements of abalone larvae during settlement and metamorphosis can be applied to improve the technology for the abalone seed production systems. An understanding and knowledge of larval development can thus lead to improved techniques and management in abalone hatchery systems. The objective of the present study was to study the larval development of *Haliotis asinina* Linnaeus.

MATERIALS AND METHODS

Conditioning of Broodstocks

The broodstocks of abalone *H. asinina* (81.4 cm in shell length, 132.6 g in weight) were naturally conditioned in 10 tons concrete race-way tanks at the Coastal Aquaculture Development Center, Prachuap Khiri Khan Province, Thailand. The water temperature was 30–33°C. A flow-through system with a flow rate of 2–3 l/min was employed. The broodstocks were fed macroalgae such as *Gracilaria fisheri*, *G. tenuistipitata* and *Acanthophora* spp. Every month, the abalone were examined for gonad maturation, which was determined by size and color according to Ebert and Houk (1984). When gonad maturation reached stage 2–3 (Ebert & Houk 1984), the abalone were transferred to the spawning room for induced spawning.

Induced Spawning

Spawning of *H. asinina* was induced during April to August, 1998, by using UV-filtered seawater. Males and females were separated in 500 L rectangular flow-through system tanks of filtered seawater with a flow rate of 2–2.5 L/min. The spawning tanks were illuminated by a 40-watt cool white fluorescent lamp (1000 lux). Each tank contained 50–60 abalone. The spawning room was set up with the following photoperiod: light (6:00 PM to 6:00 AM) and dark (6:00 AM to 6:00 PM). The abalone were reared under this reversed photoperiod for seven to ten days before spawning occurred. At 11.00 AM, the tanks were cleaned and the UV-filtered seawater was put in under a static condition. Released eggs and sperms were collected separately in a 5 L container. The fertilization period was 30 minutes after spawning. The water temperature during the fertilization period was 30–33°C.

Observation of Larval Developmental Stage

The observation of larval development of *H. asinina*, from fertilization to initiation of settlement, followed 41 stages of larval

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development described by Seki and Kan-no (1977) and Seki (1997). All experiments were performed with pooled larvae in 5-l containers until the larvae reached the last stage of larval development. Immediately after fertilization, the eggs were placed in the experimental container. Each container contained 5 eggs/ml. These were then placed under four temperature conditions: 25°C and 28° C (in controlled room temperature), 31°C and 34 C (in water baths) and at ambient temperatures of 28–35°C which served as controls. Each experiment was performed in triplicate.

The observation of larval development, carried out under a transmission compound microscope, started immediately after fertilization and was continued every five minutes until the embryos reached stage 10 or gastrula stage. After the gastrula stage, larval development was observed every 30 minutes until the embryos reached stage 41, which was the last stage of larval development. The observation of larval development continued once a day after settlement until the abalone produced the first respiratory pore. This was done in a 250 L settlement and hatchery tank containing diatom plates (*Nitzschia* sp.). The developmental stages after settlement through to the beginning of the juvenile stage followed those of Seki and Kan-no (1977) and Seki (1997).

Effects of Temperature on Larval Development

Biological Zero Point

The biological zero point is the critical water temperature at which the abalone larvae can develop completely until settlement. It was calculated from the development rate of some developmental stages (Seki & Kan-no 1977), such as hatch-out, formation of the larval retractor muscle, 90-degree torsion, formation of the epipodial tentacle and formation of the fourth tubule on the cephalic tentacles.

Effective Accumulative Temperature

The effective temperature is the difference between the water temperature and biological zero point and the quantity above the biological zero point that has an additive effect toward larval development. The summation of effective temperature value during larval development is called the effective accumulative temperature (EAT) which was calculated according to Seki and Kan-no (1977).

RESULTS

Spawning

According to the experiment, the reversal of photoperiod affected the spawning time of *H. asinina*. That is, they spawned during the day (12:00 AM–1:30 PM) instead of at night (12:00 PM–1:30 AM). The males spawned between 12:15 AM and 1:30 PM and the females spawned between 12:30 AM and 1:30 PM.

Larval Size

The eggs of *H. asinina* were dark green in color. The pigments were concentrated at the vegetal pole, while the animal pole was transparent yellowish. The average size of unfertilized eggs was 88.0 ± 8.0 μm in width, 102.4 ± 3.6 μm in length and the nuclear size of eggs was 70.4 ± 6.7 μm. After fertilization, the egg size increased; their width, length and nuclear size were 126.4 ± 17.1, 148.2 ± 10.4 and 99.5 ± 16.3 μm, respectively (Table 1). Egg size did not change until hatch-out stage. The width and length of *H. asinina* trochophore larvae were 105.3 ± 4.1 and 130.7 ± 4.8 μm, respectively (Table 1). After the larvae developed to completion of the larval shell, the size of the larval shell did not change until secretion of the juvenile shell. The first respiratory pore started building after they reached the size of 1142.4 ± 94.6 (width) × 1530.2 ± 140.7 μm (length) (Table 1).

TABLE 1.

The size of H. asinina larvae (μm), from fertilization to formation of the first respiratory pore.

Stage	Size (μm ± S.E.)	
	Width	Length
Unfertilized eggs	88.0 ± 8.	102.4 ± 3.6
Fertilized eggs to hatch out	126.4 ± 17.	148.2 ± 10.4
Trochophore larvae	105.3 ± 4.	130.7 ± 4.8
Completion of velum	108 ± 3.	135.3 ± 2.9
Larval retractor muscle	110.7 ± 4.	139.5 ± 4.2
Completion of larval shell	174.5 ± 6.	242.0 ± 6.4
Formation of operculum	175.5 ± 6.	239.8 ± 5.9
Fourth tubule on cephalic tentacle	178.3 ± 5.	245.7 ± 4.6
First day of settlement	178.8 ± 5.	247.7 ± 2.5
Beginning of first respiratory pore formation	1142.4 ± 94.	1530.2 ± 140.7

N = 30

Larval Development

The larvae of *H. asinina* had 42 stages of development (Table 2, Fig.1, Fig. 2). Shortly after fertilization (stage 1), the first polar body was discharged (stage 2) followed quickly by discharge of the second polar body (stage 3). Cleavage began after discharge of the polar bodies and development progressed to the gastrula (stages 4–11). Cilia grew along the top of the embryo forming the prototrochal girdle and apical tuft, and began beating (stage 12). The cilia caused the embryo to rotate intermittently inside the egg membrane. The stomodeum formed (stage 13) and cilia along the prototrochal girdle were completely formed (stage 14). At this stage, the embryo was classified as a trochophore larva. The larva began moving more frequently inside the egg membrane, the egg membrane became thinner, and finally burst (stage 15). The apical cilia aided the larva in bursting the egg membrane during hatch-out. The hatched larva immediately swam to the water surface.

Soon after hatch-out, the larval shell began to be secreted at the back of the larva (stage 16). The trochophore larva continued to develop until it became a veliger larva. The larva was classified as veliger when the apical region became flat and the velum was completely developed with long cilia present (stage 17).

The larval retractor muscle formed (stage 18), followed by formation of an integumental attachment to the larval shell (stage 19). The foot mass protruded from the top of the shell (stage 20) at completion of the larval shell (stage 21). During torsion, the cephalo-pedal mass rotated a 90-degree angle followed by the top of the mantle membrane tearing off from the top of the larval shell (stage 22). The velum and cephalo-pedal mass rotated relative to the region of the body covered by the larval shell. The region destined to become the mouth and the foot continued to rotate, until the cephalo-pedal mass was rotated at a 180-degree angle from its original position (stage 23).

TABLE 2.

The rate of larval development (hours) of *H. asinina* at water temperatures of 25, 28, 31, 34 C and room temperatures (28–35 C).

Sequence	Larval development stage	Time period of larval development (h)				
		25 C	28 C	31 C	34 C	28–35 C
1	Fertilization	0.00	0.00	0.00	0.00	0.00
2	First polar body	0.25	0.25	0.25	0.25	0.25–0.33
3	Second polar body	0.33	0.33	0.42	0.33	0.25–0.42
4	First cleavage (2 cells)	0.50	0.50	0.42	0.50	0.33–0.58
5	Second cleavage (4 cells)	1.00	0.92	0.83	0.92	0.75–1.00
6	Third cleavage (8 cells)	1.33	1.25	1.00	1.17	1.00–1.17
7	Fourth cleavage (12 cells)	1.42	1.33	1.25	1.25	1.17–1.33
8	Fifth cleavage (16 cells)	1.50	1.42	1.33	1.33	1.33–1.50
9	Morula (32 cells)	2.83	2.42	1.83	2.00	2.00–2.50
10	Blastula	3.83	3.17	2.83	3.00	2.50–3.00
11	Gastrula	5.50	4.00	3.50	3.50	3.00–4.00
12	Prototrochal cilia	6.00	5.00	4.17	4.00	4.50–5.00
13	Stomodeum	7.00	5.50	4.50	4.50	5.00–5.50
14	Prototrochal girdle	8.00	3.50	5.00	5.00	5.00–5.50
15	Hatch out	9.50	7.50	6.00	6.00	5.50–7.00
16	Beginning of larval shell	10.50	8.00	6.50	7.00	6.00–7.00
17	Completion of velum	12.00	9.50	8.00	8.00	7.50–8.00
18*	Larval retractor muscle	13.50	11.00	9.00	9.00	10.00–11.00
19	Integumental attachment	14.50	11.50	10.50	10.50	10.50–12.00
20	Protrusion of foot mass	16.00	12.50	10.50	10.50	11.00–12.50
21	Completion of larval shell	16.50	13.50	11.00	11.00	11.00–13.00
22*	Torsion (90° twisting)	18.00	14.00	11.50	11.50	12.00–14.00
23	180° rotation of foot mass	21.00	18.00	13.50	13.50	13.00–15.00
24	Operculum	25.00	22.50	16.00	16.00	16.00–16.50
25	Long spines on end of metapodium	29.00	23.00	18.50	17.00	18.00–19.00
26	Fine cilia on foot	30.50	25.00	20.50	20.50	19.00–20.00
27	Vertical groove in velum	35.00	27.00	21.00	21.00	21.00–22.50
28	Eye spot	40.00	28.00	21.00	21.00	21.00–23.00
29	Propodium	41.00	29.50	24.00	24.00	24.00–24.50
30	Cephalic tentacle	43.50	30.50	25.00	24.00	24.00–25.50
31	Cilia on propodium	45.00	32.00	26.50	26.00	25.00–27.00
32*	First epipodial tentacle	45.50	36.00	28.00	28.00	27.00–29.00
33	Cilia in mantle cavity	48.00	36.00	30.00	29.00	31.00–32.00
34	Apophysis on propodium	51.00	39.00	30.00	30.00	32.00–33.00
35	Otolith	55.00	41.00	34.00	33.00	32.00–33.00
36	Short spines on cephalic tentacle	57.00	42.00	34.00	34.00	33.00–34.00
37	Two tubules on cephalic tentacle	59.00	42.00	35.00	35.00	34.00–35.00
38	Third tubule on cephalic tentacle	60.00	43.00	37.00	36.00	36.00–37.00
39	Snout protrusion	62.00	45.00	39.00	38.50	38.00–39.00
40	Ciliary process in mantle cavity	64.00	46.00	40.00	38.50	39.00–41.00
41	Retractor muscle draws in mantle cavity	64.00	47.00	40.00	40.00	40.00–42.00
42*	Fourth tubule on cephalic tentacle	65.00	49.00	41.00	41.00	42.00–44.00

* the stages used for calculating the biological zero point.

There were three pairs of long spines at the posterior end of the metapodium after torsion (stage 24). The operculum formed (stage 25) and at this time, the cephalo-pedal mass could be retracted into the shell. In succession, fine cilia developed on the foot sole and began beating (stage 26), a vertical groove formed in the velum (stage 27), the eye spot appeared (stage 28), the propodium formed (stage 29), a cephalic tentacle formed on the velum (stage 30), and cilia began growing on the propodium (stage 31). Cilia formed in the mantle cavity up to the anterior edge of the velum and the cilia began beating (stage 32). The propodium twisted to the side and an apophysis appeared on the propodium (stage 33). A pair of epipodial tentacles formed on both sides of the foot under the operculum (stage 34) and crawling on a surface with its foot was now possible at this stage.

The otolith formed and was clearly visible (stage 35), short spines appeared on the cephalic tentacles (stage 36), production of the snout began from underneath the velum (stage 37), two tubules appeared on the cephalic tentacles (stage 38), a ciliary process formed on the roof of the mantle cavity (stage 39), and a third tubule formed on the cephalic tentacles (stage 40). The larval retractor muscle attached to the larval shell drew the enlarged mantle cavity toward the back of the shell (stage 41). Larval development was completed with the formation of the fourth tubule on the cephalic tentacles (stage 42).

The total time of larval development depended on the water temperature in the larval tank (Table 2). This was 65 h at 25 C, 49 h at 28°C, and 41 h at 31°C and 34 C. In the control group (28–35 C), the total time of development was 40–41 h.

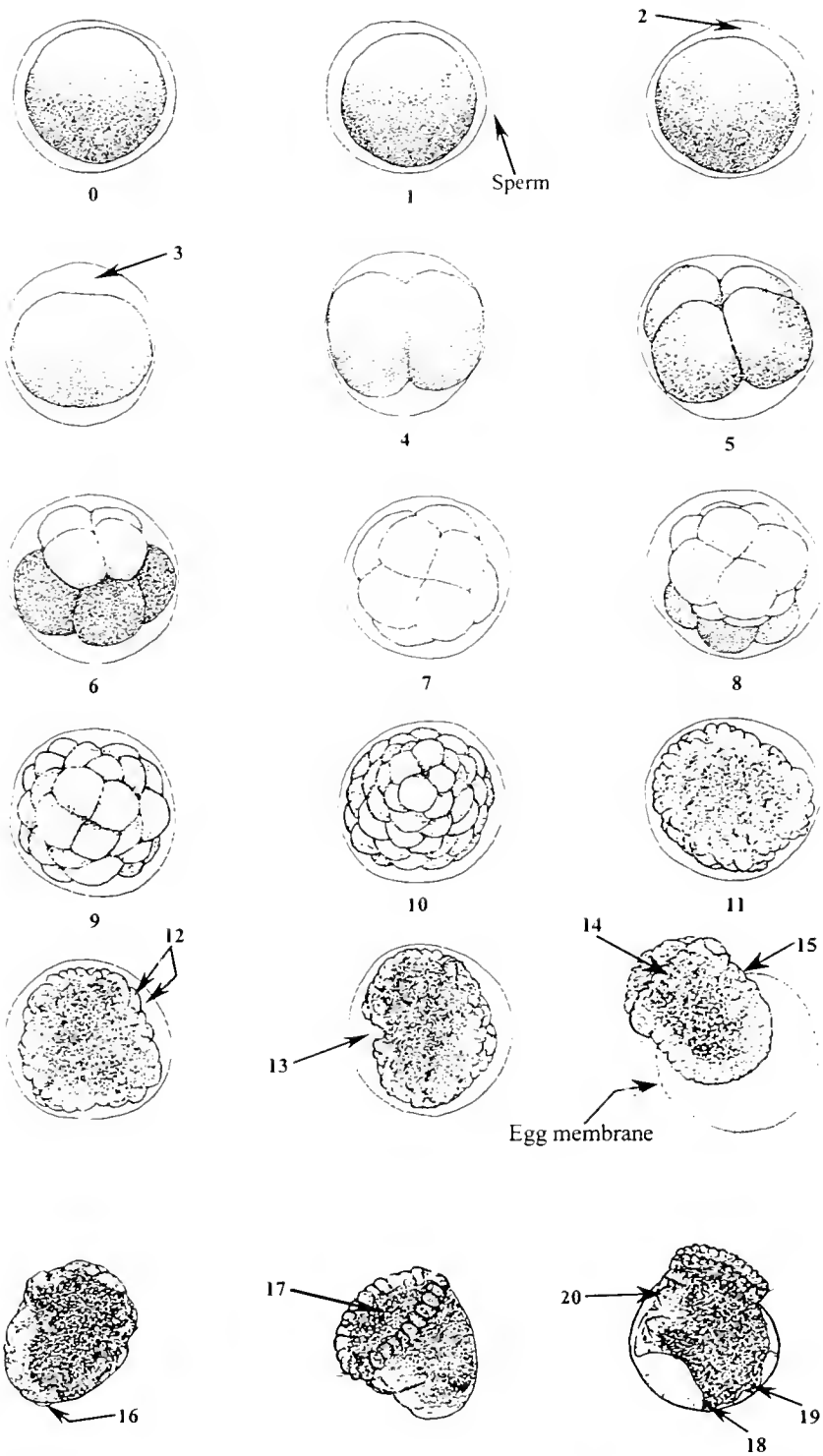

Figure 1. The development of *H. asinina* larvae from egg to the development of the foot mass. 0, unfertilized egg; 1, fertilized egg; 2, discharge of first polar body; 3, discharge of second polar body; 4, first cleavage (2 cells); 5, second cleavage (4 cells); 6, third cleavage (8 cells); 7, fourth cleavage (12 cells, top view); 8, fifth cleavage (16 cells); 9, morula; 10, blastula; 11, gastrula; 12, appearance of prototrochal girdle and cilia along the prototrochal girdle*; 13, appearance of stomodeum (rotation); 14, prototrochal girdle and cilia along the prototrochal girdle are completely formed.*; 15, hatch - out; 16, beginning of larval shell formation; 17, the apical region becomes flat and the velum is completely developed with long cilia present; 18, appearance of larval retractor muscle; 19, appearance of integumental attachment; 20, development of foot mass. * There are 24 cilia surrounding the velum along the prototrochal girdle.

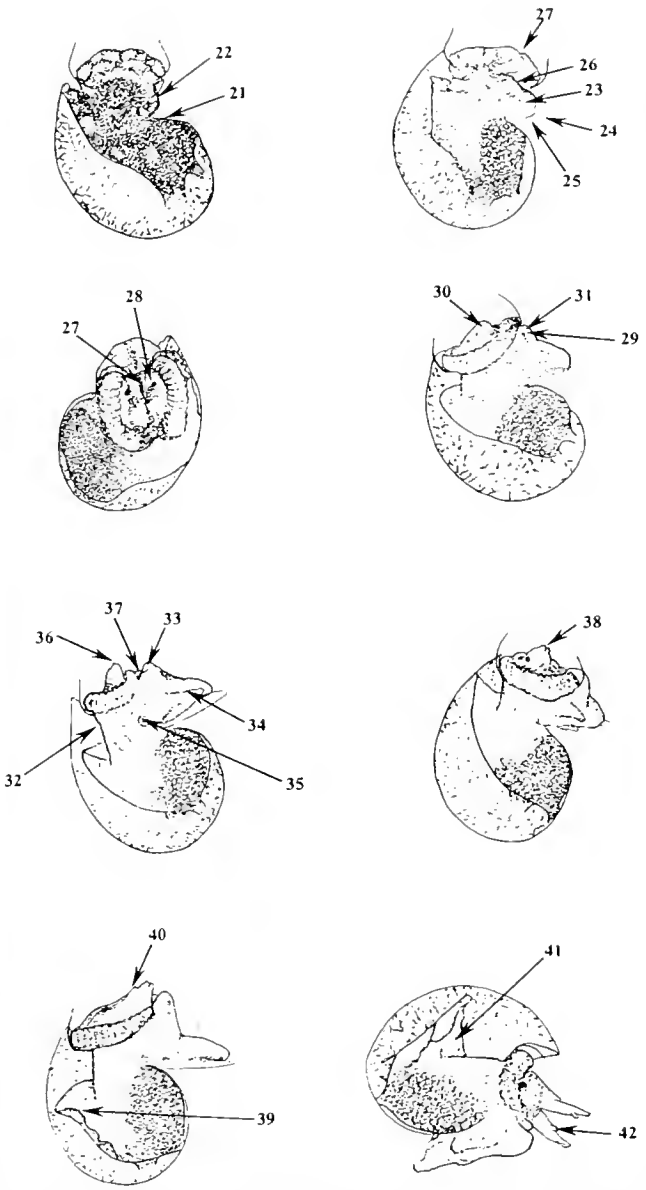

Figure 2. The development of *H. asinina* larvae from the completion of larval shell stage to the appearance of the fourth tubule on cephalic tentacles. 21, completion of larval shell; 22, cephalo-pedal mass 90 degree torsion; 23, foot muscle 180 degree torsion; 24, appearance of spine on end of metapodium; 25, operculum; 26, appearance of fine cilia on foot sole; 27, vertical groove formation in velum; 28, appearance of eye spot; 29, appearance of propodium; 30, appearance of cephalic tentacles; 31, appearance of cilia on the propodium; 32, appearance of cilia in mantle cavity up to the anterior edge of the velum; 33, formation of apophysis on propodium; 34, formation of first epipodial tentacle; 35, appearance of otolith; 36, appearance of spines on cephalic tentacles; 37, protrusion of snout underneath the velum; 38, appearance of two tubules on cephalic tentacle; 39, ciliary process forms on the roof of the mantle cavity; 40, third tubule appearance on cephalic tentacle; 41, the retractor muscle attached to the larval shell draws the enlarged mantle cavity toward the back of the shell; 42, fourth tubule appearance on cephalic tentacles.

After 44 hours, or when the larvae had already formed the fourth tubule on the cephalic tentacles, they were placed in the settlement tank. On the first day of settlement, they were still in the exploration and inspection stage. They were repeatedly swimming with the velum and temporarily crawling over the surface of the diatom plate. On the second day, most larvae were shedding the velum and the mantle membrane moved to the shell edge. Some larvae had already secreted the new shell. The settled larvae had already formed the fifth and sixth tubules on the cephalic tentacles and the ciliated process was already formed near the right cephalic tentacle. At this stage, all were crawling on the plate. In the mantle cavity, the cilia on the roof of the mantle were beating to circulate the water and functioned as a ctenidium. However, some larvae still had the velum and were still in the exploration and inspection stage. On the third day of settlement, the juvenile shell increased in length. The post-larvae began to feed on diatoms that were smaller than 10 μm. On the fourth day, the heart began to form and was beating. On the roof of the mantle was a prominent structure that developed into the ctenidium. The juvenile shell increased in length. On day eight, the second pair of epipodia began to form and the number of tubules on the cephalic tentacles increased. The eye stalks were completely developed. From day nine to day 24 of the post-larvae stage, the number of ctenidia and epipodia increased. The juvenile shell increased in size. The first respiratory pore began to form on day 24 to day 30.

Effects of Water Temperature on Larval Development

Biological Zero Point

The biological zero point was calculated from the developmental rates of hatch-out, formation of larval retractor muscle, 90 degree torsion, formation of epipodial tentacles and the fourth tubule on the cephalic tentacle stage of *H. asinina* at temperatures of 25°C, 28°C, 31°C, and 34°C. In this experiment, the biological zero point of the larval development rate at 34°C was not calculated because it was close to that of the development at 31°C. The average biological zero point of *H. asinina* was 15.0 ± 0.3°C (Table 3).

Effective Accumulative Temperature

The important stages of larvae in the aspect of abalone aquaculture management are hatch-out, completion of larval shell, formation of the first epipodial tentacle and the fourth tubule on the cephalic tentacles (Hahn 1989a). The effective accumulative temperatures at water temperatures of 25°C, 28°C, 31°C, and 34°C were 95, 98, 96 and 114°C-hour, respectively, in the hatch-out stage; 165, 176, 176 and 209°C-hours, respectively, in the comple-

TABLE 3.

Equation of relationship between water temperature and time inversion of beginning formation of the fifth larval developmental stage (1/time) and biological zero point of the important larval stages of H. asinina.

Larval developmental stage	Equation of relationship	Biological Zero Point
Hatch out	$1/t^a = 0.0102T^b - 0.1515$	14.9
Larval retractor muscle	$1/t = 0.0073T - 0.1091$	14.9
Torsion (90° twisting)	$1/t = 0.0052T - 0.0768$	14.8
First epipodial tentacle	$1/t = 0.0023T - 00356$	15.5
Fourth tubule on cephalic tentacle	$1/t = 0.0015T - 0.0220$	14.7
	Average	15.0 ± 0.3

[a] t = hours, [b] T = °C

tion of the larval shell stage; and 650, 637, 656 and 779.0°C-hours, respectively, in the formation of the fourth tubule on cephalic tentacle stage (Table 4). From observation, a high incidence of abnormal development and mortality was found in larvae reared in water higher than 31 C.

DISCUSSION

The pigments of abalone eggs were derived from maternal yolk and they were retained through the trochophore and veliger larvae. The color of abalone eggs and larvae varies among species. They were dark green in *H. asinina*, brown in *H. fulgens* Philippi, beige in *H. sorenseni* Bartsch, olive in *H. corrugata* Wood (Hahn 1989a), violet in *H. coccinea canarensis* Nordsieck (Harrison & Grant 1971) and green or blue-green in *H. cyclobates* Péron & Lesueur, *H. laevigata* Donovan, *H. scalaris* Leach and *H. roei* Gray (Shepherd & Laws 1974). In some abalone species, the color of the eggs varies in each stage of development, such as in *H. rubra* Leach (Shepherd & Laws 1974). The pigments in the eggs of *H. asinina* are concentrated at the vegetal pole, while the animal pole is transparent yellowish. This feature is in contrast with those of other abalone species, where pigmentation of the eggs is dark at the animal pole and light at the vegetal pole (Hahn 1989a). In *H. coccinea canariensis*, the animal pole is violet and the vegetal pole is slightly yellowish (Harrison & Grant 1971). The pigments in *H. asinina* also appear in the visceral mass, so that the visceral mass

TABLE 4.

The effective accumulative temperature (EAT) of larval development of *H. asinina* at water temperatures of 25, 28, 31, 34 C and room temperatures (28–35 C).

Sequence	Larval development stage	HEAT (°C – h)				
		25 C	28 C	31 C	34 C	Mean
1	Fertilization	0.0	0.0	0.0	0.0	0.0
2	First polar body	2.5	3.3	4.0	4.8	3.6
3	Second polar body	3.3	4.3	6.7	6.3	5.1
4	First cleavage (2 cells)	5.0	6.5	6.7	9.5	6.9
5	Second cleavage (4 cells)	10.0	12.0	13.3	17.5	13.2
6	Third cleavage (8 cells)	13.3	16.3	16.0	22.2	16.9
7	Fourth cleavage (12 cells)	14.2	17.3	20.0	23.8	18.8
8	Fourth-fifth cleavage (12–16 cells)	15.0	18.5	21.3	25.3	20.0
9	Morula (32 cells)	28.3	31.5	29.3	38.0	31.8
10	Blastula	38.3	41.2	45.3	57.0	45.4
11	Gastrula	55.0	52.0	56.0	66.5	57.4
12	Prototrochal cilia	60.0	65.0	66.7	76.0	66.9
13	Stomodeum	70.0	71.5	72.0	85.5	74.8
14	Prototrochal girdle	80.0	45.5	80.0	95.0	75.1
15	Hatch out	95.0	97.5	96.0	114.0	100.6
16	Beginning of larval shell	105.0	104.0	104.0	133.0	111.5
17	Completion of velum	120.0	123.5	128.0	152.0	130.9
18	Larval retractor muscle	135.0	143.0	144.0	171.0	148.3
19	Integumental attachment	145.0	149.5	168.0	199.5	165.5
20	Protrusion of foot mass	160.0	162.5	168.0	199.5	172.5
21	Completion of larval shell	165.0	175.5	176.0	209.0	181.4
22	Torsion (90° twisting)	180.0	182.0	184.0	218.5	191.1
23	180° rotation of foot mass	210.0	234.0	216.0	256.5	229.1
24	Operculum	250.0	292.5	256.0	304.0	275.6
25	Long spines on end of metapodium	290.0	299.0	296.0	323.0	302.0
26	Fine cilia on foot	305.0	325.0	328.0	389.5	336.9
27	Vertical groove in velum	350.0	351.0	336.0	399.0	359.0
28	Eye spot	400.0	364.0	336.0	399.0	374.8
29	Propodium	410.0	383.5	384.0	456.0	408.4
30	Cephalic tentacle	435.0	396.5	400.0	456.0	421.9
31	Cilia on propodium	450.0	416.0	424.0	494.0	446.0
32	First epipodial tentacle	455.0	468.0	448.0	532.0	475.8
33	Cilia in mantle cavity	480.0	468.0	480.0	551.0	494.8
34	Apophysis on propodium	510.0	507.0	480.0	470.0	516.8
35	Otolith	550.0	533.0	544.0	627.0	563.5
36	Short spine on cephalic tentacle	570.0	546.0	544.0	646.0	576.5
37	Two tubules on cephalic tentacle	590.0	546.0	560.0	665.0	590.3
38	Third tubule on cephalic tentacle	600.0	559.0	592.0	684.0	608.8
39	Snout protrusion	620.0	585.0	624.0	731.5	640.1
40	Ciliary process in mantle cavity	640.0	598.0	640.0	731.5	652.4
41	Retractor muscle drawn in mantle cavity	640.0	611.0	640.0	760.0	662.8
42	Fourth tubule on cephalic tentacle	650.0	637.0	656.0	779.0	680.5

of larvae is dark green, while the foot, velum and cephalic parts are transparent yellowish. *H. sorenseni* larvae are beige in color, while the velar margin is yellowish (Hahn 1989a). In some abalone species, the pigments seem to be concentrated at the velum: for example, *H. fulgens* larvae are generally brown with green velar margins, *H. corrugata* larvae are light yellow-green with a velar fringe of a darker shade of green (Leighton 1974), *H. coccinea canariensis* is orange in visceral mass and violet in foot, velum and cephalic mass (Harrison and Grant 1971).

The size of fertilized abalone eggs also varies from species to species. The diameter of the fertilized egg of *H. asinina* was 125.4 × 148.2 μm. It is 103 μm in *H. coccinea canariensis* (Pena 1986), 150–225 μm in *H. cyclobates* (Shepherd & Laws 1974), 200–250 μm in *H. laevigata* (Shepherd & Laws 1974), 200 μm in *H. sorenseni* (Leighton 1972) and *H. rubra* (Harrison & Grant 1971), 210 μm in *H. tuberculata* Linnaeus (Koike 1978), 230 μm in *H. discus* Reeve (Ino 1952) and *H. iris* Gmelin (Harrison & Grant 1971), 270 μm in *H. gigantea* Gmelin (Ino 1952) and 280 μm in *H. seiboldii* Reeve (Ino 1952) (Table 5). The size of the trochophore and veliger also varies depending on species (Table 5).

There are 41 stages of larval development in *H. discus hannai* (Seki & Kan-no 1977), *H. diversicolor supertexta* Lischke (Oba 1964), *H. sieboldii* (Ino 1952) and *H. discus* (Ino 1952). Only 32 stages were described in *H. rufescens* Swainson (Hahn 1989a). There are 42 stages of larval development in *H. asinina*. The previous studies on larval development of several species of abalone showed that there were no differences in the development from fertilization to trochophore larva in *H. discus hannai* (Seki & Kan-no 1977), *H. sieboldii* (Ino 1952), *H. discus* (Ino 1952) and *H. diversicolor supertexta* (Oba 1964). However, there are certain differences between larvae of these abalone species and that of *H. asinina*. In other abalone species, the fourth cleavage produced 16 cells, while 12 cells were observed in the fourth cleavage (stage 7) and 16 cells were observed in the fifth cleavage (stage 8) of *H. asinina*. *H. asinina* larvae had both long and short cilia on the prototrochal cell (stage 12), while larvae of other abalone species had only long cilia. In addition, *H. asinina* trochophores appeared to lack the apical tuft cilia that were reported in the trochophores of other abalone species.

The larval development of *H. asinina*, from trochophore stage to torsion, is similar to that of *H. discus hannai*, *H. sieboldii*, *H. discus* and *H. diversicolor supertexta*. The larval retractor muscle (stage 18) was formed prior to formation of the integumental attachment to the larval shell (stage 19). In addition, protrusion of the foot mass (stage 20) and development of the operculum (stage 24) occurred after these stages (Seki & Kan-no 1977, Ino 1952, Oba 1964). Similarly, *H. asinina*, *H. discus hannai*, *H. sieboldii*, *H. discus* and *H. diversicolor supertexta* formed cilia during early larval development and the cilia were retained (Seki & Kan-no 1977).

There are many differences in larval development from torsion to metamorphosis. As in *H. discus hannai*, the larval shell of *H. asinina* was completed (stage 21) before torsion (stage 22) occurred (Seki & Kan-no 1977). In *H. sieboldii*, Ino (1952) found that torsion occurred first and then the larval shell was completed about 13 hours later. In *H. asinina*, the spine at the end of propodium (stage 29) was very fine and very difficult to observe. It could be seen after the formation of the operculum. In *H. discus hannai*, the spine was found at the end of the propodium before the formation of the operculum. In addition, like *H. sieboldii*,, the spines on the metapodium (stage 25) of *H. asinina* were formed after the operculum (Ino 1952). In *H. discus hannai*, these spines were formed before the operculum (Seki & Kan-no 1977).

In *H. asinina*, the cilia in the mantle cavity (stage 33) up to the anterior edge of the velum were seen after the formation of the first epipodial tentacle (stage 32). In *H. discus hannai*, the formation of the first epipodial tentacle occurred before the appearance of apophysis and cilia in the mantle cavity. In addition, in *H. asinina*, the ciliary process on the roof of the mantle cavity (stage 40) was formed after the formation of the third tubule on the cephalic tentacles (stage 38). In *H. discus hannai*, the third tubule on the cephalic tentacles was seen before the formation of the ciliary process on the roof of the mantle cavity.

H. asinina and *H. discus hannai* developed all the larval stages from stage 34 to 42 (*H. asinina*), or stage 32 to 41 (*H. discus hannai*) before metamorphosis. As in *H. discus hannai*, the otolith (stage 35) was observed after the formation of the cephalic tentacle (Seki & Kan-no 1977).

The duration of the pelagic stage in gastropods is variable, and the larvae may spend from two to 14 days in the water column (Leighton 1974). Also the abalone larvae are pelagic and the duration of development varies from species to species. In general, tropical abalone species such as *H. asinina* and *H. diversicolor*

TABLE 5.

The size of egg, trochophore and larva of some *Haliotis* spp.

Species	Egg (μm)	Trochophore (μm)	Completion of larval shell (μm)	References
H. asinina	126 × 148	105 × 131	175 × 242	The present study
H. coccinea canariensis	103	201 × 160	264 × 206	Pena (1986)
H. cyclobates	150–225			Shepherd and Laws (1974)
H. laevigata	200–250			Shepherd and Laws (1974)
H. discus	230	178 × 200	290	Ino (1952)
H. rufescens	—	160 × 195	210 × 270	Ebert and Houk (1984)
H. sieboldii	280			Ino (1952)
H. gigantea	270			Ino (1952)
H. iris	230			Harrison and Grant (1971)
H. rubra	200			Harrison and Grant (1971)
H. sorenseni	200			Leighton (1972)
H. tuberculata	210			Koike (1978)

supertexta usually have a short larval development (Oba 1964). In *H. asinina*, the time durations for hatch-out, torsion and formation of the first epipodial tentacle were 6.0, 11.5 and 28.0 h, respectively (at 31°C) and 7.5, 14.0 and 36.0 h, respectively (at 28°C) (Table 6). These durations are quite similar to those in *H. diversicolor supertexta*, *i.e.*, 6.0, 13.0 and 38.0 h for hatch-out, torsion and formation of the first epipodial tentacle, respectively (at 26.2°C) (Owen et al. 1984) (Table 6). The first epipodial tentacle of *H. asinina* appeared before the formation of the apophysis on the propodium, and the larvae started to crawl on the bottom after the formation of the apophysis. In *H. discus hannai* larvae, the apophysis on the propodium was formed before the appearance of the first epipodial tentacle and the larvae started to crawl on the bottom when the first epipodial tentacle was formed (Seki & Kan-no 1977).

The induction of settlement could be done in *H. asinina* after the formation of the apophysis on the propodium. However, the larvae were not able to metamorphose at this stage until they reached the last stage of development. The abalone larvae are competent to metamorphose when they have four branches on the cephalic tentacles, the fully developed foot is able to pull the larvae upright and they can move by ciliary action (Seki & Kan-no 1977, Ebert & Houk 1984). The duration of complete larval development of *H. asinina* was 41.0 to 65.0 h or only 1.7 to 2.7 days (Table 6), while the duration of larval development of temperate abalone species was from 99.0 to 360.0 h or 4.1 to 15 days depending on the species and water temperature (Table 6). The duration of larval development in *H. asinina* at water temperatures of 31°C and 34°C was identical. However, from observation, water temperature at 34°C is rather high and may cause abnormal development and high mortality of larvae. The optimum water temperature of larval development in *H. asinina* was 28°C to 31°C.

Temperature is an important factor in many stages of development such as gonad maturation, spawning (Hahn 1989b, Hahn 1989c) and larval development (Seki & Kan-no 1977, Hahn 1989a). Normally, the animals grow faster in a higher temperature. Larval development in *Haliotis* spp. also occurs at a faster rate at higher water temperature (Hahn 1989a). The development of *H. asinina* larvae at water temperature of 31°C (41 h) was faster than that at 25°C (65 h). In *Haliotis rufescens* Swainson, the hatching time in water temperature of 17°C to 18°C was faster than that at 15°C (Table 6).

A successful culture and high yield are assured by changing the rearing conditions at three important stages during larval development: hatch-out, development of larval shell, and settlement (Hahn 1989a). So the information on the biological zero point and the effective accumulative temperature (EAT) is necessary for hatchery management. The biological zero point varies among abalone species, depending on water temperature in each location (Hahn 1989a). For example, the biological zero point of *H. rufescens* is 0.9°C which is below the ambient water temperature (Hahn 1989a). In *H. asinina*, the biological zero point (15.0°C) is also below the ambient water temperature (20°C).

Biological zero point is one of the values used to calculate EAT. The average EAT of larval development of *H. asinina* is lower than those of temperate abalone species such as *H. discus hannai* and *H. rufescens* (Table 7). The important EAT values in hatchery are during hatch-out, completion of larval shell formation and competency of settlement stage. After hatch-out, the healthy trochophore larvae will swim toward the surface immediately and can be collected near the water surface and transferred to the other tank (Shepherd 1975). The trochophore larvae of *H. asinina* should be decanted as soon as possible after they reach the EAT 101°C-hours, in order to reduce bacterial contamination from decomposition of egg membranes and unfertilized eggs. Moreover, the enzymes secreted by the larvae to facilitate hatching may contribute to the lowering of water quality (Hahn 1989a). From the trochophore stage to completion of the larval shell stage, the larvae

TABLE 6.

The time of hatch out, 90° torsion, first epipodial tentacle formation and fourth tubule on cephalic tentacle formation of several abalone species.

Species	Temperature (°C)	Hatch out (h)	Larval retractor muscle (h)	Torsion (90° twisting) (h)	First epipodial tentacle (h)	Fourth tubule on cephalic tentacle (h)	References
H. discus hannai	20	12.6	23.4	32.1	64.9	99	Seki and Kan-no (1977)
H. discus	16–17	20		45–46	120		Hahn (1989c)
H. sieboldii	16–17	18	29	35	96		Ino (1952)
H. gigantea	16–18	21–22		40–43			Ino (1952)
H. rufescens	15	20				144	Ebert and Houk (1984)
	15	20				175	Hahn (1989c)
	17	24		44			Leighton (1974)
	17	25	36	48			Hahn (1989c)
	18	16–24					Owen et al. (1984)
H. fulgens	17	14		48		360	Leighton (1974)
H. corrugata	16	17		48		192	Leighton (1974)
H. sorenseni	15	24				240	Leighton (1972)
H. tuberculata	20	13	20.5		84	120	Koike (1978)
H. coccinea canariensis	15	21		52			Pena (1986)
H. diversicolor supertexta	26.2	6		13	38		Oba (1964)
H. asinina	25	9.5	13.5	18.0	45.5	65.0	The present study
	28	7.5	11.0	14.0	36.0	49.0	The present study
	31	6.0	9.0	11.5	28.0	41.0	The present study
	34	6.0	9.0	11.5	28.0	41.0	The present study

TABLE 7.

The biological zero point (°C) of some *Haliotis* spp.

Species	Biological zero point (°C)	References
H. discus hannai	7.6	Seki and Kan-no (1977)
H. discus	8.5	Hahn (1989c)
H. gigantea	9.0	Seki and Kan-no (1977)
H. rufescens	8.5	Seki and Kan-no (1977)
H. rufescens	0.9	Hahn (1989c)
H. fulgens	9.9	Leighton (1974)
H. asinina	15.0	The present study

are very fragile, because they do not have a protective organ. The water in the tank should be changed after formation of the larval shell, or when the EAT of larval development reaches 181.4°C-hours. The EAT of settlement stage of *H. asinina* larvae was 680.5°C-hours when the larvae reached the formation of the third tubule on the cephalic tentacles. Settlement occurs rapidly on wavy-plate substrata if the larvae are healthy and competent to settle and metamorphose (Hahn 1989a).

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ISOLATION AND GROWTH OF EIGHT STRAINS OF BENTHIC DIATOMS, CULTURED UNDER TWO LIGHT CONDITIONS

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ABSTRACT We isolated seven strains of benthic diatoms from three abalone commercial fishery areas in the state of Baja California and in the proximity of several hatcheries and nursery. In all the cases, we isolated several strains of the genus *Nitzschia* and one strain of *Amphiprora paludosa var. hyalina*. Monospecific and non-axenic cultures of each strain were maintained on batch cultures under white and blue light at high (150 μE m^{-2} s^{-1}) photon fluence rates. For all the diatoms we observed that the growth rate (μ) in the second or third day of culture had high values of duplicated strains per day. We compared the growth rate of these strains with a control specie *Navicula incerta*, a strain used as food in several abalone farms in México, and found similar growth rates in some of the cases. For each strain, a covariance analysis was used to compare growth using white versus blue light wavelength. No significant differences in growth were observed between the two light conditions. We obtained high growth rate for *Amphiprora paludosa var. hyalina* with almost double values of those obtained with of the control species *N. incerta* and the others strains: *N. thermalis var. minor*, *N. fonticola var. pelagica* and *N. frustulum var. perminuta*. The sub-strains of *N. laevis* exhibit equal growth rate and nearly 50% less than that of *N. incerta*. Our results suggest that some strains of benthic diatoms offer a good potential for use in feeding abalone post-larvae, because they can be cultured under high light irradiance apparently without photoinhibition and may exhibit high growth rates and high cell concentrations.

KEY WORDS: benthic diatoms, growth rates, light quality, abalone, post-larvae.

INTRODUCTION

Benthic diatoms play an important role in abalone cultures, acting as inductors for settlement of the larvae and serving as the main source of nourishment during the early juvenile stage (Ebert & Houk 1984, Kawamura et al. 1995). To be used as nourishment in abalone cultures: the diatoms must be preferably of a size less than 10 μm, exhibit adequate nutritional level, and adhere strongly to the substrate so that they may be readily ingested by the abalone post-larvae and juveniles (Hahn 1989, Fleming et al. 1996, Kawamura et al. 1998).

Rearing of post-larva abalone under culture conditions relies on two different strategies to obtain the first microalgae food supply. The first method involves allowing the proliferation of natural populations of phytoplankton. This culture method has the disadvantage of being unpredictable, and results in uncontrolled species introduction and densities. The growth of the postlarvae may be insufficient or inadequate when natural populations of diatoms or other phytoplankton species are used, due to the seasonal variability that they present (Ebert & Houk 1984, Kawamura & Hirano 1992, Voltolina 1994). The second method uses controlled condition cultures of one or several benthic diatom species. This strategy has the advantage that the food densities can be controlled producing a more predictable growth rate, and a higher survival of abalone post-larvae. However, in some cases the species of benthic diatoms used in abalone culturing farms are non-endemic to the region, which may result in the introduction of species and the displacement or succession of local species. Therefore, it is important for farms to use benthic diatoms isolated from the area, to guarantee the quantity and quality of nourishment while avoiding the ecological unbalance of the organisms of the region.

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Algae cultures may be influenced though the manipulation of environmental variables. Light is the driving force of photosynthesis. As such, its quantity and spectral composition and its fluctuation within the culture control biomass production rates (Dubinsky et al. 1995). The potential of this light-induced manipulation of the cultures has not been sufficiently explored in the context of aquaculture. Surprisingly, only few studies have used light manipulation to obtain different growth rates and nutritional values of plankton microalgae in culture (Flaak & Epifanio 1978, Correa-Reyes 1993, Sánchez-Saavedra & Voltolina 1994, Sánchez-Saavedra & Voltolina 1995).

The effect of light irradiance on the growth rate of benthic diatoms has been poorly studied (McBride 1990, Flores-Vergara 1998). It is generally considered that the light intensity and quality suitable for rapid growth, is related to the type of habitat utilized in the natural environmental. Usually for benthic diatoms culture for abalone post-larvae only a low light intensity is supplied, and in abalone farms, light is provided with fluorescent lamps at 80 μE m^{-2} s^{-1} on the water surface for less than a 12- hour period (Hahn, 1989). In several studies, the light regimen used for the culture of benthic diatoms was between 20 and 200 μE m^{-2} s^{-1} (Thompson et al. 1992, Cota-Sánchez 1998, Roberts et al. 2000).

In the natural environment, the spectral characteristics of light changes as function of depth, red light is the first wavelength that is absorbed in the water column. In the benthic environment, blue light is the light quality that is usually available and hence used by photosynthetic organisms. These are the conditions found in the habitat shared by benthic diatoms and abalone. Surprisingly, there is no previous work, relating the effect of light quality on growth of benthic diatoms; all studies have been conducted on planktonic diatoms (Gostan et al. 1986, Senger 1987).

The aim of this study was to compare the growth rate of several strains of benthic diatoms species isolated from different zones

close to abalone farms in México, and to culture these under two different light conditions: white and blue light.

MATERIALS AND METHODS

We collected biofilm samples of benthic diatoms from three different regions of fishery production and within the proximity of abalone farms: two in Baja California (Ejido Eréndira and Isla de Cedros) and one in Baja California Sur (Puerto Nuevo) in México (Fig. 1). The isolation techniques used to obtain the different species or strains of benthic diatoms, involved micropipetting and serial dilution as described by Hoshaw and Rosowski (1973) and by Voltolina (1994). The different benthic diatom strains were preselected based on morphology and attachment capacity. During the isolation process of the different stocks and with the purpose of eliminating bacteria and fungi, baths were applied with a mixture of 50 ppm penicillin and 100 ppm streptomycin during an eight-hour period.

For the taxonomic identification of the diatoms, we proceeded to eliminate the organic matter present in the cultures of each diatom strain. While preserving the ornamental structures by washing with concentrate H_2SO_4, K_2HPO_4 1M, 3% glutaraldehyde and different percentages of ethanol in a sequence of 10, 25, 50, 75, 90 and 100 (Nalewajko 1989). An aluminum film permanent slide was mounted for observation under scanning electron microscope (SEM) for each diatom strain. Phase contrast (100×) and planapochromatic (63×) microscopy was used in the taxonomic analysis. Taxonomic criteria were based on morphology of the frustule, using the classic literature and recent works (Navarro 1981a, Navarro 1981b, Navarro 1982, Navarro 1983, Round et al. 1990, Simonsen 1987, Siqueiros-Beltrones 1994, Siqueiros-Beltrones 1999, Siqueiros-Beltrones 2000, Siqueiros-Beltrones & Sánchez-Castrejón 1999, Siqueiros-Beltrones & Voltolina 2000). Curatorial records on cards for each taxon were made, including SEM photographs. The permanent slides were included in the "Diatom Collection of the Museo de Historia Natural" of the Universidad Autónoma de Baja California Sur, México.

We also used as control species a strain of *Navicula incerta* isolated from coastal waters of Bahía de Todos Santos, México by the Microalgae Laboratory staff of the "Instituto de Investigaciones Oceanológicas" Universidad Autónoma de Baja California, México. That strain was obtained from the abalone farm "Abulones Cultivados S. de R.L. de C.V." in Ejido Eréndira, Baja California, México.

Non-axenic monospecific batch cultures of each strain were placed in ten replicate 250-ml Erlenmeyer flasks with 150 ml of Guillard and Rhyther's (1962) "f" medium. The densities of the inoculum used to grow each strain varied as a result of differences in cell size (Table 1). However for comparison purposes, each one of the strains of diatom species on the experimental condition of light received the same quantity of inoculum. The culture conditions were salinity 34±1 ‰ and temperature 22 ± 1°C provided by air conditioning. These values are similar to those used in laboratory abalone production. The pH was not controlled and values ranged between 8.2 and 9.7.

Every other day we checked the cell concentrations. Ultrasound was applied to the culture flasks for fifteen to sixty seconds, depending on the age of culture, to dislodge the cells that were firmly attached to the walls (Voltolina 1985). Two flasks from each light condition and strain were used to determine cell concentrations; these were discarded to avoid the possible effect of the ultrasonic method on the growth of the diatoms. The evaluation of cell concentration was measured through direct counts using a haemacytometer. The growth rate and generation time was determined by

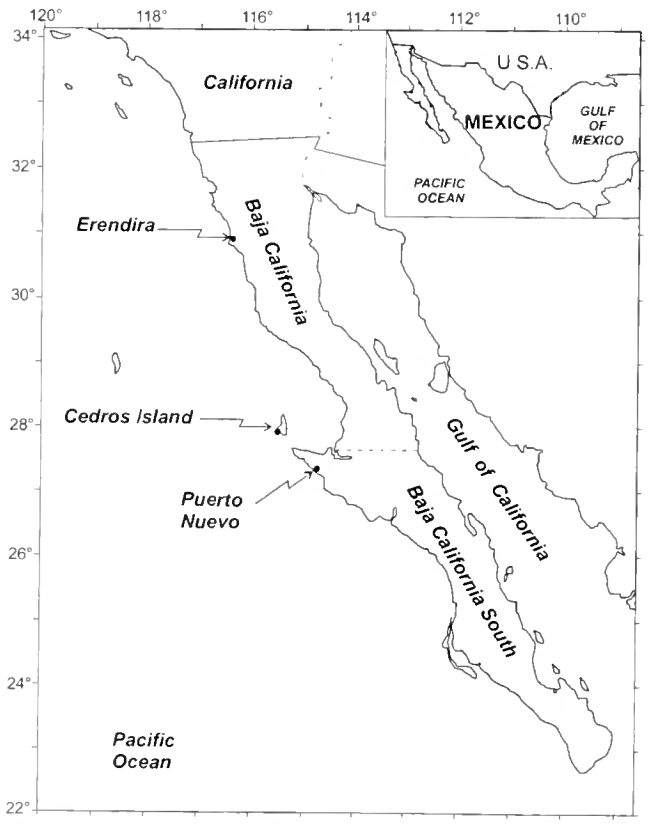

Figure 1. View of different localities sampled to obtain benthic diatoms strains in Ejido Eréndira, Cedros Island on Baja California, and Puerto Nuevo, in Baja California Sur, México.

TABLE 1.

Benthic diatom strains isolated from three different zones close to abalone farms in Baja California (B.C.) and Baja California Sur (B.C.S.), México. Procedence, isolation techniques and average size in microns (length and width).

Strain	Procedence	Isolation Techniques	Size (microns)
Nitzschia thermalis var. minor (A)	Ejido Eréndira, B.C.	Micropipette	21.0 × 7.5
Nitzschia laevis (B)	Ejido Eréndira, B.C.	Micropipette	7.5 × 6.0
Nitzschia laevis (C)	Puerto Nuevo, B.C.S.	Serial dilution	8.0 × 5.5
Nitzschia laevis (D)	Puerto Nuevo, B.C.S.	Serial dilution	7.0 × 4.0
Navicula incerta (E)	Ejido Eréndira, B.C.	Serial dilution, Donated	14.5 × 5.5
Nitzschia cf. fonticola var. pelagica (F)	Isla de Cedros, B.C.	Serial dilution	16.4 × 4.3
Amphiprora paludosa var. hyalina (Donk.) Reimer *(G)*	Isla de Cedros, B.C.	Micropipette	13.2 × 4.6
Nitzschia frustulum var. perminuta (H)	Isla de Cedros, B.C.	Serial dilution	10.3 × 3.6

calculations the $\log_2$ cell concentration at different times for each strain as described by Sorokin (1973).

We exposed each strain to two continuous light conditions. The first one was obtained using normal laboratory Cool White 40 W fluorescent lamps (Sylvania F40CW) that provided a spectral emission with two main peaks of emission principally, one in the blue-green wavelengths (453–500 nm) and the other within the red (550–620 nm). In the second, ambient Blue Light was provided with 40 W General Electric F40B, and the peak of emission was primly within the blue-green light (430–470 nm). For the two light qualities, a photon fluence rate of 150 $\mu E\ m^{-2}\ s^{-1}$, because this irradiance is commonly used in several commercial abalone hatcheries in México. Photon fluence rates values were measured at the center of the culture surface with a QSL-100 (Biospherical Instruments) 4 π quanta meter. The spectral characteristics of the lighting sources were determined as described by Sánchez-Saavedra and Voltolina (1996a).

A covariance analysis and two ways ANOVA were used to compare cell concentration among treatments and culture days. A p-level of 0.05 was used to evaluate significance with the "Statistica 5.0 for Windows" program (StatSoft Inc., 1996).

RESULTS

As result of the sample collections, six stocks of benthic diatoms were isolated (Stocks: A through G, Fig. 2, Fig. 3, Fig. 4, Fig. 5), and one strain previously isolated was obtained by donation. In the majority of the localities, stocks belonging to the *Nitzschia* genus were isolated. The other one was identified as *Amphiprora paludosa var. hyalina*. Size ranged between 7 and 20 μ (Table 1).

In all cases, the mean cell concentration for a given strain grown under white or blue light conditions (Table 2), did not exhibit significant differences A: $p = 0.971$, $F = 0.001$; B: $p = 0.231$, $F = 1.534$; C: $p = 0.070$, $F = 3.695$; D: $p = 0.120$, $F = 2.644$; E: $p = 0.491$, $F = 0.494$; F: $p = 0.231$, $F = 1.534$; G: $p = 0.070$, $F = 3.695$). Due to differences in length and width of the cells, we obtained different cell concentrations between the eight strains. However the highest cell densities (for white and blue light respectively) were for *Amphiprora paludosa var. hyalina* and the size of this strain was one of the highest (48.4 and 44.58 10^5 cell ml^{-1}), two substocks of *Nitzschia laevis* (4.85 and 41.43 10^5 cell ml^{-1} and 37.98 and 53.94 10^5 cell ml^{-1}), and *Navicula incerta* (30.64 and 28.98 10^5 cell ml^{-1}). The lowest cell concentrations were found for *Nitzschia thermalis var. minor* (2.50 and 3.02 10^5 cell ml^{-1}) (Table 2), and the strain with the largest cell size (21.0 $\times$ 7.5 μ) (Table 1).

The mean cell concentration values for each of the eight benthic diatoms cultures under white or blue light for ten days, yielded significant differences in the covariance analysis (for white light $p = 0.000$; $F = 25.662$ and for blue light $p = 0.000$; $F = 25.075$). The growth rate for each strain was similar (Table 3) and with equal values under the two light treatments examined. The highest growth rates occurred during the first two days of culture. We obtained high growth rate values for *Amphiprora paludosa var. hyalina* (2.70 and 2.69), which was twice as high as that found for the control species *Navicula incerta* (1.62 and 1.58). Similar values were obtained for *N. thermalis var. minor*, *N. fonticola var. pelagica* and *N. frustulum var. perminuta* (1.18 to 1.62). For the sub-strains of *N. laevis*, we measured the same growth rate for each one; the values were nearly 50% less than that for *Navicula incerta*.

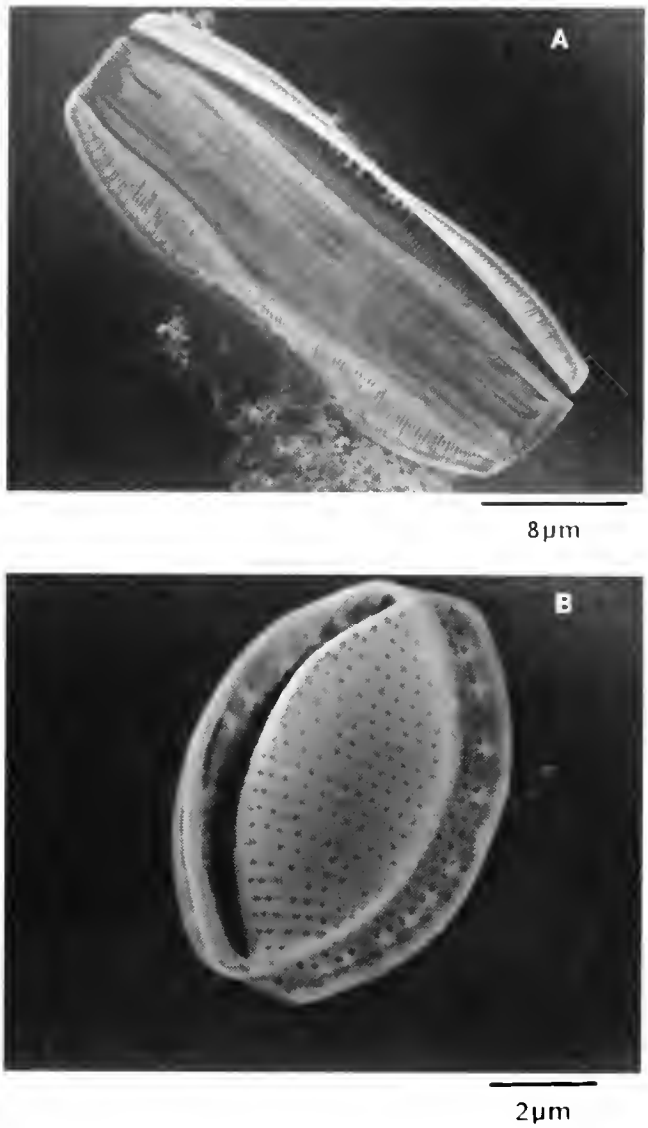

Figure 2. Images of *Nitzschia thermalis var. minor* (A) and *Nitzschia laevis* (B), isolated close to the post-larvae abalone farm Abulones Cultivados S. de R.L. de C.V. in Ejido Eréndira, Baja California, México.

Under the two light conditions, the generation time obtained for the eight strains as expected were lower than or close to one for the first two days and increased as corresponded to cell concentrations values. The lower value of generation time under white or blue light for the first days (0.37 for day two and 2.04 for day four), confirmed the higher cell concentration values for *Amphiprora paludosa var. hyalina* (Table 4). For days six to ten for both light conditions and for the eight strains, the inconsistency with the values of generation time, were due to minimum values of differences on cell concentrations on the last days of culture.

DISCUSSION

For the selection of benthic diatom species with a potential to be used in nutrition of abalone post-larvae, several suitable characteristics have to be considered. Among the suggested ones are: an adequate size, many authors recommend sizes around 10 μ, the degree of adhesion, the frustulum width or degree of silicification

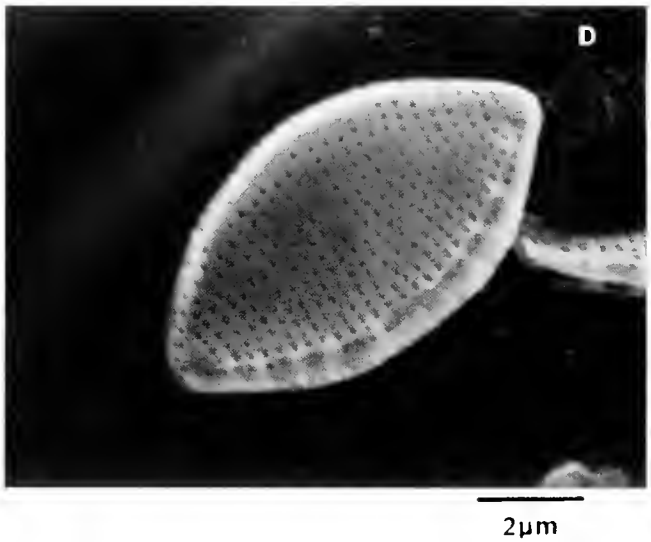

Figure 3. Images of *Nitzschia laevis* (C) and *Nitzschia laevis* (D), isolated close to the laboratory for abalone seed production SCPP Emancipación in Puerto Nuevo, Baja California Sur, México.

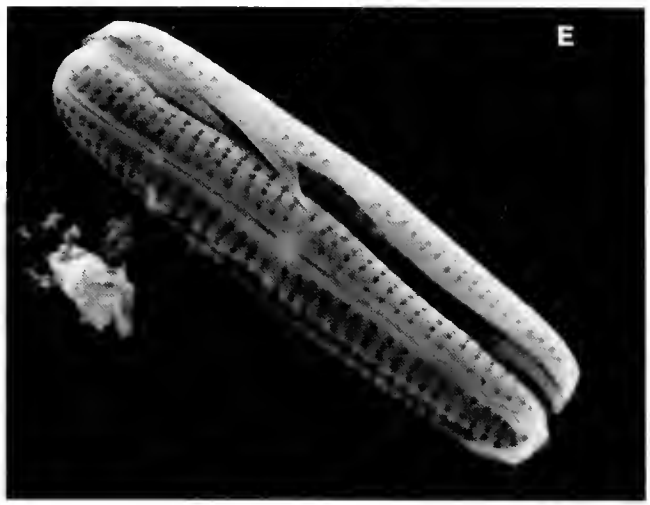

Figure 4. Images of *Navicula incerta* (E) isolated from coastal waters of Bahía de Todos Santos by the staff of microalgae laboratory of the Instituto de Investigaciones Oceanológicas of the Universidad Autónoma de Baja California, México. For this work this strain was obtained from the abalone farm Abulones Cultivados S. de R.L. de C.V. in Ejido Eréndira, Baja California, México.

and motility capacity (Kawamura et al. 1998). However for other cell sizes, apparently the abalone radula can cause the cell to become deformed and create physical rupture of the diatom cell, as described by Daume et al. (1997), who observed that abalone post-larvae settling over coralline algae were capable of breaking the theca of *Cocconeis* spp., and were capable of better assimilating the cellular content. This ability of the radula of abalone post-larvae is corroborated by the work of Roberts et al. (1999). In our results, the lowest values of cell concentrations corresponded to largest cell size, e.g. *Nitzchia thermalis var. minor*. Smaller cell sizes corresponded to medium cell concentrations, e.g. the three different strains of *Nitzschia laevis*. The highest cell concentrations and largest cell sizes corresponded to *Amphiprora paludosa var. hyalina* and *Nitzschia frustulum var. perminuta*.

We can isolated six strains of *Nitzchia* spp., because several *Nitzschia* species have fragile cellular walls and in some cases strong adhesion to substrates, making them susceptible to grazing by abalone post-larvae (Siqueiros-Beltrones, 1999). In addition Siqueiros-Beltrones, 1999 suggested that the diatoms may break when grazed, and lead to a better assimilation of the cellular material.

However, for the eight diatom strains isolated in this study there is no current information available regarding degree of silicification, adhesion and digestibility by abalone post-larvae. It has been proposed that the species of the *Nitzschia* genus may provide good nourishment for abalone post-larvae due to their high growth rates and dominance in successions in culturing systems (Siqueiros-Beltrones, 1999). In addition, the substratum from which benthic diatoms were isolated should be considered (Siqueiros-Beltrones 2000, Siqueiros-Beltrones & Voltolina 2000). The habitat were they are isolated determines the hardness of cellular walls. All the strains isolated for this study, were obtained of zones with lowest water movement or from tanks on abalone farms.

In this work, we observed high growth rates for all species during the first two days of culturing. Other studies (Cota-Sánchez 1998, Cuevas-Rocha 1998, Flores-Vergara 1998), recorded maximum growth rates between days four and five for benthic diatoms of the genera *Nitzschia*, *Amphora* and *Navicula*. However, for other benthic diatoms as such *Navicula cf. cincta* and *Nitzschia sp.* cultured individually or mixed with *Amphora cf. catenula*, the maximum growth rates were observed between days two and three. These studies proposed that the release of extracellular products could inhibit or promote growth (Cota-Sánchez 1998, Cuevas-Rócha 1998). Since these studies and ours used different species and culture conditions (light, nutrients, temperature, pH, salinity, and water movement), the growth rates obtained can be influenced directly. Thus, we propose that culture conditions are an important fact than must be considered, for explaining different growth rates and consequently differences on chemical composition and nutritional value.

Several abalone nurseries discarded the use of benthic diatoms when the mean generation time was higher than one day (Voltolina 1985, Voltolina 1994, Cuevas-Rócha 1998). All the diatom strains

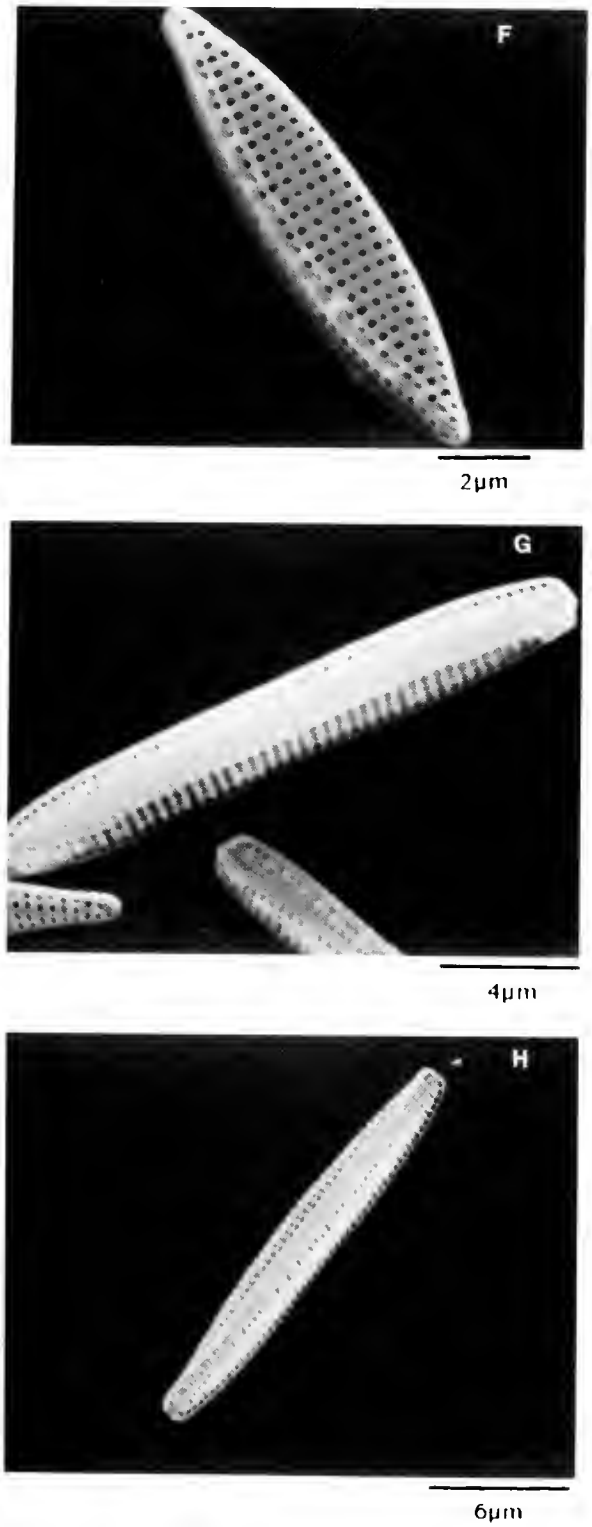

Figure 5. Images of *Nitzschia cf. fonticola var. pelagica* (F), *Amphiprora paludasa var. hyalina* (G) and *Nitzschia frustulum var. perminuta* (H), isolated near the laboratory for abalone seed production SCPP Pescadores Nacionales de Abulón in Isla de Cedros, Baja California, México.

used on this work had mean generation time closest to one, by this we can recommend using the eight isolated strains and experiment on this work for abalone postlarvae nutrition. High variations on generation time are due to the minimum difference obtained on cell concentration values. However, The sample strategy used may produce lower differences on cell concentration values due to minimum differences between Erlenmeyer flask culture replications.

Since light provides the energy that drives photosynthesis other parameters such as photoperiod, light energy or light intensity, and the light quality must be considered. Some algae adapt to very low light intensities and can live at depths as much as 200 m (Fallu 1991). No previous works about the effect of light quality on benthic diatoms or abalone ethology are available. However, it should be considered that research on this subject is needed to understand the ecology and physiological response of alga communities (macro and micro) and of abalone. Abalone have negative phototaxis and is usually found in refuges or cavities in their natural environment or in culture tanks (Hahn 1989).

Controlling the level of light available to the culture tanks has been the most important and most effective means of influencing the alga community on which abalone post-larvae feed. Commonly high light levels produce filamentous algae, this originates displacement of benthic diatom species and occasional loss of postlarva with the water circulation system exchange. Usually this practice is accomplished by utilizing greenhouse shading material, or black polyethylene plastic (McMullen & Thompson 1989). Depending on cell density, pigmentation and cell geometry, the optical density and the wavelength of light can be changed within the culture, depending on the light source of vessel configuration and pond depth (Dubinsky et al. 1995).

Benthic diatoms may be prolific at great depths <1% irradiance (Cahoon et al. 1993), where the main component of the light spectrum is blue wavelength. Abalone also dwells under low light conditions were benthic diatoms serve as their main food source *in situ*, especially for their postlarvae and juveniles.

However, other works reported good biomass production at high irradiance (around 300 $\mu E\ m^{-2}\ s^{-1}$) (Kromkamp et al. 1998). It was determined recently that they are in fact not reaching their optimal biomass production under low light conditions. The saturation light intensity may vary among diatom species. The results presented here suggest that light intensity was not limiting for growth, because we obtained good growth rates when compared to other reports for other benthic diatoms (McBride 1990, Simental-Trinidad 1999). With an adequate light quality we believe growth rate can be increased and metabolic changes induced by effect of deviation of carbon metabolism and cell number or biomass quantity increased as described for plankton diatoms (Flaak & Epifanio 1978, Sánchez-Saavedra & Voltolina 1996b). However, for the eight diatoms, strains cultured under blue light for this work, high growth rates or increase biomass production was not obtained compared with white light. These important differences in physiologic response could be related to the vertical natural distribution of benthic diatoms, and other factors may be the efficiency of light availability, that can be assumed different for each species according to its preference. The results obtained in this study differ from those reported by Sánchez-Saavedra and Voltolina (1994, 1996a, 1996b), who kept cultures of *Chaetoceros* sp. under blue and white light and observed significant differences in growth, finding greater cellular concentrations when cells were cultured under white light.

TABLE 2.

Mean values (n = 3) of cell concentration (1×10^5 cell ml^{-1}) and standard deviation (in parenthesis) cultured throughout 10 days with white (a) and blue light (b) for *Nitzschia thermalis var. minor* (A); *Nitzschia laevis* (B); *Nitzschia laevis* (C); *Nitzschia laevis* (D); *Navicula incerta* (E); *Nitzschia cf. fonticola var. pelagica* (F); *Amphiprora paludosa var. hyalina* (G) and *Nitzschia frustulum var. perminuta* (H).

a

Time (days)	Cell concentration of benthic diatoms (1×10^5 cell ml^{-1})							
	A	B	C	D	E	F	G	H
0	0.30 (0.00)a	3.00 (0.00)bd	3.00 (0.00)bd	3.00 (0.00)bd	0.90 (0.00)b	2.00 (0.00)b	0.45 (0.00)c	3.00 (0.00)cd
2	1.93 (0.43)a	9.10 (0.51)bd	9.51 (0.57)bd	9.83 (0.04)bd	8.51 (0.98)b	14.70 (0.64)b	18.96 (0.00)c	18.45 (2.08)cd
4	1.89 (0.60)a	19.61 (4.56)bd	10.17 (0.36)bd	16.99 (0.18)bd	12.37 (0.10)b	20.75 (0.01)b	37.46 (0.73)c	28.49 (1.00)cd
6	3.31 (0.85)a	24.69 (0.81)bd	22.54 (2.49)bd	28.70 (5.59)bd	13.99 (1.56)b	21.35 (0.42)b	28.35 (0.00)c	34.37 (0.15)cd
8	2.44 (0.24)a	33.09 (8.14)bd	31.20 (6.15)bd	36.21 (2.67)bd	21.65 (8.89)b	22.15 (0.77)b	46.38 (4.67)c	30.74 (2.18)cd
10	2.50 (0.08)a	31.68 (3.22)bd	34.85 (3.80)bd	37.98 (5.18)bd	30.64 (1.47)b	26.45 (2.69)b	48.40 (8.32)c	37.18 (2.69)cd

b

Time (days)	Cell concentration of benthic diatoms (1×10^5 cell ml^{-1})							
	A	B	C	D	E	F	G	H
0	0.30 (0.00)a	3.00 (0.00)b	3.00 (0.00)bc	3.00 (0.00)bcd	0.90 (0.00)b	2.00 (0.00)b	0.45 (0.00)c	3.00 (0.00)bcd
2	2.04 (0.27)a	10.28 (0.69)b	12.69 (0.17)bc	15.37 (0.89)bcd	8.06 (0.01)b	12.18 (2.44)b	18.65 (0.10)c	17.69 (0.69)bcd
4	2.29 (0.55)a	16.91 (1.06)b	13.57 (1.54)bc	20.44 (3.29)bcd	14.90 (2.21)b	18.62 (0.57)b	35.63 (2.69)c	28.44 (0.81)bcd
6	2.31 (0.57)a	19.98 (4.71)b	32.64 (12.18)bc	26.19 (2.12)bcd	13.53 (1.40)b	27.28 (1.25)b	35.44 (7.00)c	32.29 (1.91)bcd
8	2.45 (0.92)a	24.90 (5.17)b	30.55 (3.84)bc	32.73 (0.08)bcd	17.12 (1.12)b	26.53 (3.08)b	41.51 (1.01)c	32.82 (0.26)bcd
10	3.02 (0.41)a	35.15 (2.79)b	41.43 (4.50)bc	53.94 (10.35)bcd	28.98 (0.00)b	25.23 (1.45)b	44.58 (0.65)c	41.88 (1.73)bcd

Some letters indicate lack of significant differences as determined with a two-way ANOVA and Duncan a *posteriori* test $\alpha = 0.05$, a < b < c.

TABLE 3.

Group rates (n = 3) throughout ten culture days under white (a) and blue light (b) for *Nitzschia thermalis* var. *minor* (A); *Nitzschia laevis* (B); *Nitzschia laevis* (C); *Nitzschia laevis* (D); *Navicula incerta* (E); *Nitzschia cf. fonticola var. pelagica* (F); *Amphiprora paludosa var. hyalina* (G) and *Nitzschia frustulum var. perminuta* (H).

a

Time (days)	Growth rate of benthic diatoms (divisions by day)							
	A	B	C	D	E	F	G	H
2	1.33	0.80	0.83	0.86	1.62	1.44	2.70	1.31
4	−0.02	0.54	0.05	0.40	0.27	0.25	0.49	0.32
6	0.41	0.18	0.57	0.37	0.09	0.02	−0.21	0.14
8	−0.21	0.20	0.23	0.17	0.29	0.03	0.35	−0.08
10	0.02	−0.02	0.08	0.03	0.28	0.13	0.03	0.14

b

Time days	Growth rate of benthic diatoms (divisions by day)							
	A	B	C	D	E	F	G	H
2	1.38	0.89	1.04	1.18	1.58	1.30	2.69	1.28
4	0.08	0.36	0.05	0.20	0.44	0.31	0.47	0.34
6	0.00	0.11	0.61	0.18	−0.07	0.28	−0.01	0.09
8	0.03	0.16	−0.02	0.16	0.14	−0.02	0.12	0.01
10	0.17	0.26	0.22	0.35		−0.03	0.05	0.17

TABLE 4.

Generation time throughout ten culture days under white (a) and blue light (b) for *Nitzschia thermalis var. minor* (A); *Nitzschia laevis* (B); *Nitzschia laevis* (C); *Nitzschia laevis* (D); *Navicula incerta* (E); *Nitzschia cf. fonticola var. pelagica* (F); *Amphiprora paludosa var. hyalina* (G) and *Nitzschia frustulum var. perminuta* (H).

a

Time (days)	Generation time of benthic diatoms (days)							
	A	B	C	D	E	F	G	H
2	0.76	1.25	1.20	1.17	0.62	0.70	0.37	0.77
4	2.98	1.98	21.95	2.53	3.82	4.05	2.04	3.22
6	2.44	14.24	1.76	2.88	18.43	65.40	−4.74	7.53
8	−5.61	10.23	8.24	15.50	5.08	18.44	2.89	−15.79
10	−5.02	0.71	−4.85	15.60	6.56	8.53	−1.45	7.29

b

Time (days)	Generation time of benthic diatoms (days)							
	A	B	C	D	E	F	G	H
2	0.73	1.13	0.96	0.85	0.63	0.78	0.37	0.78
4	28.36	2.88	−22.04	5.35	2.35	3.72	2.16	2.96
6	−377.06	−9.88	1.73	5.77	5.63	3.63	2.56	14.45
8	−0.28	−6.19	0.37	6.58	7.88	27.92	82.45	−23.41
10	12.43	4.20	4.55	3.06	2.41	−15.54	23.18	6.06

One other important consideration is, all the isolated strains are conditioned to local environmental fluctuations. Usually, many of the strains kept in laboratories are rarely suited to cooler environments and many of the algae are planktonic, therefore the disadvantage of poor culture conditions or lack on benthic habitat of abalone post-larvae. It is often considered desirable to have pure strains of microalgae. This may allow farmers complete control over what they feed their spat and controlled experimentation or commercial production. It is not generally desirable to feed a single species of microalgae, several pure strains can be mixed to improve the nutrition of abalone post-larvae (Fallu, 1991). Consideration of the chemical composition as protein, carbohydrates and lipid content of the strains, and abalone post-larvae requirements, could lead to the production of a more nutritional food value from diatoms promoting higher survival and growth rates of abalone.

The eight strains of diatoms used in this study offer a good alternative to be used in the nutrition of abalone post-larvae. These strains are acclimated to local environmental fluctuations (temperature and light principally). However, it is necessary to evaluate the nutritional value of these local diatom strains for abalone post-larvae, and relate their biochemical composition, adhesion to the substrate and digestibility with respect to the survival and growth of abalone post-larvae.

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BIOCHEMICAL COMPOSITION OF BENTHIC MARINE DIATOMS USING AS CULTURE MEDIUM A COMMON AGRICULTURAL FERTILIZER

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ABSTRACT Three strains of benthic marine diatoms (*Nitzschia thermalis var. minor, Nitzschia laevis* and *Navicula incerta*) were grown individually in batch systems with 10 liters of non-conventional culture media formulated with three common agricultural fertilizers. The quantity and quality of the biomass produced using non-conventional culture media were compared to those obtained with a traditional culture media, "f/2". The objective of this study was to consider a low cost and alternative media to culture the benthic diatoms commonly used in commercial abalone hatcheries. No significant differences were found among the biomass produced with non-conventional or control media. The trend in chemical composition of each diatom culture had a significant difference during 10 days of experimentation for all species. For the cultures of diatoms *Nitzschia thermalis var. minor, Nitzschia laevis* and *Navicula incerta*, the chemical composition did not differ between media. Our results suggested non-conventional media did not affect biomass or biochemical composition and it cost eight times less than "f/2".

KEY WORDS: Benthic diatoms, agricultural fertilizer, chemical composition, mass culture

INTRODUCTION

When undertaking laboratory research on molluscs, and/or when commercially producing these, large quantities of microalgal biomass are needed as the main source of food required for their development and normal growth (Fabregas et al. 1987). Benthic diatoms play an important role in abalone culture, acting as inductors for larval settlement, and as food for the early juvenile stages before these can ingest macroalgae (Searcy-Bernal et al. 1992, Kawamura et al. 1995).

The biochemical composition of microalgae is dependent on numerous factors, including the nutrient concentration and composition of the growth medium (Fabrégas et al. 1986, Wilkfors 1986), temperature (Thompson et al. 1992), light intensity and wavelength (Figueroa et al. 1996, Sánchez-Saavedra & Voltolina 1996), photoperiod (Sicko-Goad & Andersen 1991) and growth stage at time of harvest (Dunstan et al. 1991). It can, therefore, be substantially altered by manipulating their culture conditions (Dubinsky et al. 1978, Ben-Amotz et al. 1987, Geldenhuys et al. 1988). Generally, where benthic diatoms are used in the production of abalone postlarvae, the nutrient enrichment of culture medium is not used to avoid increase of production costs (Ebert & Houk 1989). However, algal biomass production obtained is substantially less than that produced within an enriched medium. To obtain high quantities of diatom biomass of suitable nutritional quality, the development of microalgae cultures using alternate or non-conventional simplified and economical culture media becomes necessary. One possible source of economical nutrients for microalgae cultures is agricultural fertilizers. They have been used successfully to culture *Isochrysis aff. galbana* and *Chaetoceros mulleri* (Valenzuela-Espinoza et al. 1999).

One important consideration in the formulation of culture media is that they must have an adequate proportion of nutrients in relation to the nutritional requirements of the microalgae (Valenzuela-Espinoza 1997). It is also important that the production of benthic diatoms be of high density and of good nutritional quality for the abalone postlarvae. All this should be achieved at low cost (De la Cruz & Alfonso 1975, González-Rodríguez & Maestrini 1984). The culture media prepared with laboratory grade reagents are expensive (Simental-Trinidad 1999). It is also not practical to use these for aquacultural purposes since the conditions of commercial culture differ from laboratory conditions. One possible solution to this problem is to use the more economical agricultural fertilizers as culture media (González-Rodríguez & Maestrini 1984, Simental-Trinidad 1999).

Commercial production of mollusc larvae implies the production of massive cultures of high quality microalgae, since these are indispensable for the adequate nourishing of larvae, postlarvae or adults. This is where a "bottle neck" is found in commercial farms. Thus, several alternatives are being sought to decrease the production costs of culture media for microalgae. The aim of this work was to evaluate the chemical composition of three benthic diatoms *Nitzschia thermalis var. minor, Nitzschia laevis* and *Navicula incerta* cultured in an alternative media made with commercial agricultural fertilizer as a supplement of nitrogen, phosphate and silica compounds required to grow diatom cultures.

MATERIALS AND METHODS

For this work, we used the benthic diatoms *Nitzschia thermalis var. minor* (29.57 μ), *N. laevis* (7.56 μ) and *Navicula incerta* (12.82 μ) (Fig. 1). Both *Nitzschia* species are potentially useful in abalone aquaculture and were isolated from production farms in the locality of Ensenada, Baja California, México (Correa-Reyes et al. *in preparation* b). *Navicula incerta* (12.82 μ) was isolated in coastal waters off Bahía Todos Santos, México by the staff of the Microalgae Laboratory of the "Instituto de Investigaciones Oceanológicas, Universidad Autónoma de Baja California, México". The strain of *Navicula incerta* was obtained from the abalone farm "Abulones Cultivados", Ejido Eréndira, Baja California, México.

The commercial medium "f/2" (Guillard 1975) was used as the control culture medium in all the experiments. Nutrients available from non-conventional cultured medium lacked trace metals, and vitamins were added in an equal concentration to that indicated in the formulation of the control medium "f/2" (Table 1). We used liquid agricultural fertilizers for the preparation of the non-conventional medium. The fertilizers were obtained from "Insumos y Servicios Agrológicos S.A. de C.V.", Ejido Camalú, Baja California, México. Nitrogen density was 1.37 grl^{-1}, total nitrogen

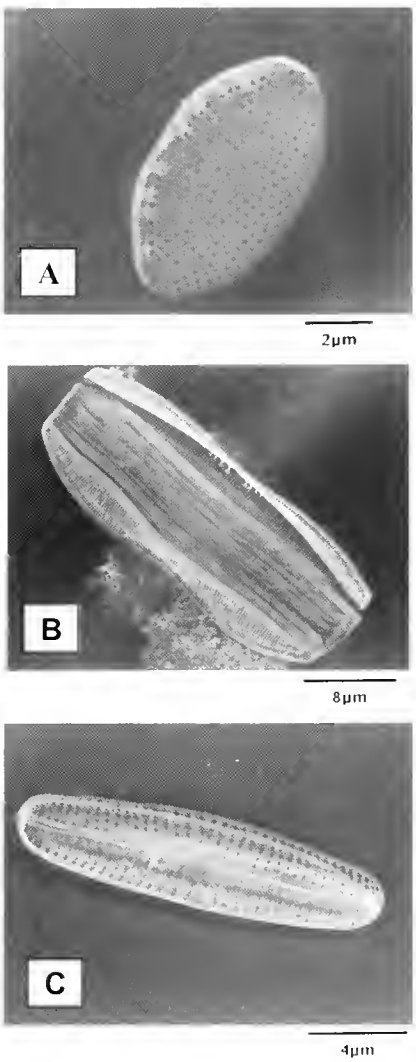

Figure 1. Benthic diatoms *Nitzschia laevis* (A), *Nitzschia thermalis var. minor* (B) and *Navicula incerta* (C).

was 32% and the cost US$0.39 l^{-1}. Phosphorous density was 1.69 grl^{-1}, total phosphorous was 72% of phosphoric acid and the cost US$0.78 l^{-1}. Silica was as sodium methasilicate with 0.107 moles l^{-1} and was obtained from a distributor "Química Internacional de Tecate S.A. de C.V.", México, at US$0.85 l^{-1}.

Cultures were prepared from the production of inoculum in progressive volumes of 10 ml, 250 ml and 900 ml. Each of the three species was acclimated to the cultured medium before experimentation. These cultures were maintained under controlled light conditions (150 μEm^{-2} s^{-1}), environmental temperature (21 ± 1 °C) and pH (between 8.7 and 9.0).

Batch cultures were kept in circular 18 l plastic containers of 38 cm by 30 cm. White plastic containers were used for the assay. The containers were provided with a lexon acrylic lid with two orifices, one for the incoming air and the other for the outcoming air. Air was not injected to the culture medium and it was only used on the surface to create a circulation cell to maintain a constant temperature. The seawater for the culture medium was disinfected chemically following the methodology described by Hemerick (1973).

A sampling strategy, without sampling replacement, was made for biomass quality and quantity, due to the benthic diatoms characteristics and the presence of a biofilm on the bottom of the container. Sampling for each species and culture medium consisted of taking two containers every 48 hours and harvesting the whole culture to determine the proximal composition and biomass in triplicate. To determine microalgal biomass, microalgae cells were counted in a 0.1-mm depth haematocytometer with the aid of a compound microscope.

For the proximal analyses, the microalgae were harvested from each container, concentrated and placed in ultrasound water to eliminate possible lumps. Subsamples were taken from the concentrate and were reconcentrated in Poretics GF-C glass fiber filters (2.5 cm diameter) previously rinsed with distilled water, and incinerated at 470 °C for four hours. After filtering, the samples were stored at −20 °C as recommended by Cordero-Esquivel et al. (1993). The analytical technique used for total and ash free dry weights was as those described by Sorokin (1973). Samples were dried at 60 °C to constant weight in a conventional oven, and ashed in a muffle furnace at 470 °C for eight hours.

Analysis of proteins were performed by varying the extraction time and NaOH concentrations for optimum extraction, as proposed by Correa-Reyes et al. (*in preparation* a). Protein determination was undertaken using the protocols proposed by Lowry et al. (1951). Carbohydrates were extracted following the Whyte method (1987) and their determination was made according to the methodology of Dubois et al. (1956). Lipids were extracted following the methodology of Bligh and Dyer (1959) and their quantification was estimated by the method of Pande et al. (1963). Calibrations curves were obtained for each one of the analyses following the indications described by the colorimetric techniques specified for each case, using a Shimadzu spectrophotometer model UV-1201. Bovine albumin (98%) was employed as a protein standard, glucose (99%) as a carbohydrate standard and tripalmitin (99%) as a lipid standard.

We used a covariance statistical analysis to determine differences between the productions obtained within different culture media and the three strains.

RESULTS

Nitzschia laevis

The percentage of protein content for *Nitzschia laevis* (Table 2) cultured in "f/2" medium, had a maximum (31.257) on the eighth day of culture, while that cultured in the experimental media showed the highest protein percentage (25.470) on the fourth day. There were significant differences in the protein percentage between both treatments ($F = 18.821$, $P = 0.000$). There were no significant differences in carbohydrate ($F = 2.726$, $P = 0.115$) and lipid percentage ($F = 0.878$, $P = 0.360$) for this strain cultured on the experimental medium.

Organic dry weight and ash content increased significantly with culture age and were similar to those obtained when the diatom were grown on the experimental medium (Table 2).

Nitzschia thermalis *var.* minor

Nitzschia thermalis var. minor (Table 3), showed a maximum protein percentage (33.505) on the fourth day of culture in the control medium. The microalgae cultured in the experimental medium reached the maximum protein percentage (35.579) on the second day of culture. There were no significant differences between both treatments ($F = 3.233$, $P = 0.084$).

TABLE 1.

Chemical composition and cost for 1000-l of prepared culture media used to grow benthic diatoms. Agricultural fertilizers were used as experimental media and "f/2" media as Guillard and Ryther (1975) for control medium.

Source	Agricultural Fertilizer Chemical form	Ionic form	Cost (US $)	"f/2" Medium Chemical form	Ionic form	Cost (US $)	(μmol)
	Nitrate	NO_3					
Nitrogen	Ammonium	NH_4	3.09×10^{-5}	Sodium nitrate	$NaNO_3$	1.01×10^{-2}	
	Urea	$CO(NH_4)_2$					882.0
Phosphorus	Phosphoric acid	H_3PO_4	2.26×10^{-6}	Sodium phosphate	$NaH_2PO_4 \cdot H_2O$	5.90×10^{-4}	36.3
Silica	Sodium methasilicate	$Na_2SiO_3 \cdot 9H_2O$	4.25×10^{-4}	Sodium methasilicate	$Na_2SiO_3 \cdot 9H_2O$	6.80×10^{-4}	107.0

Source	Common chemicals for the two media Chemical form	Ionic form	Cost (US $)	(μmol)
	Ferric chloride	FeEDTA	1.05×10^{-3}	23.3000
	Copper sulphate	$CuSO_4 \cdot 5H_2O$	1.10×10^{-6}	0.0395
Trace metals	Zinc sulphate	$ZnSO_4 \cdot 7H_2O$	2.30×10^{-6}	0.0765
	Cobaltous chloride	$CoCl_2 \cdot 6H_2O$	1.90×10^{-6}	0.0425
	Manganese chloride	$MnCl_2 \cdot 4H_2O$	2.14×10^{-5}	0.9000
	Sodium molibdate	$Na_2MoO_4 \cdot 2H_2O$	1.23×10^{-5}	0.0260
	Biotin	$C_{10}H_{16}N_2O_3S$	4.20×10^{-7}	1.336×10^{-6}
Vitamins	Cianocobalamin	$C_{63}H_{88}CoN_{14}P$	3.90×10^{-7}	0.393×10^{-6}
	Thiamin	$C_{12}H_{17}ClN_4OS$	4.90×10^{-9}	0.18726

The carbohydrate content in microalgae cultured in the control medium had a maximum percentage (23.923) on the second day of culture, while the experimental group did not show significant differences along the whole culture period. There were differences between both treatments only on the second day of culture in which the cultured microalgae in the control medium obtained the highest percentages (Table 3) ($F = 7.694$, $P = 0.012$).

The lipid percentage in the experimental culture showed the highest values (21.06) on the tenth day of culture, while the cultured microalgae in the experimental medium showed the highest values from the sixth (34.247) to the tenth day (44.549). There were significant differences between both treatments only on the tenth culturing day, on which the cultured microalgae in the control medium had a higher percentage ($F = 6.791$, $P = 0.015$).

The fraction of organic dry weight and ash content showed a similar trend to those of *Nitzschia laevis*. Their final concentrations were also similar to those obtained for that species (Table 3).

Navicula incerta

Navicula incerta showed a maximum protein percentage during days two (35.153) and four (35.405) when cultured in the control medium, whereas when cultured in the non-conventional medium, maximum percentage was obtained during the second day (33.530)

TABLE 2.

Average proximal composition (expressed in percentage) based on the dry weight organic of *Nitzschia laevis* cultured in 18-l plastic containers with "f/2" culture media (Guillard & Ryther 1975) and with agricultural fertilizers.

Culture medium	Day	Cell ml^{-1}	% Proteins	% Carbohydrates	% Lipids	% Ash
	2	144687a	22.533a	5.310a	15.907a	60.560a
		(30937)	(0.71)	(0.49)	(1.48)	(7.25)
	4	374100b	25.625a	7.074a	18.339a	54.121a
"f/2" medium		(96638)	(1.68)	(0.48)	(1.62)	(7.55)
	6	739450b	27.221a	8.676a	13.183a	46.016a
		(136000)	(0.11)	(0.85)	(1.29)	(4.23)
	8	1133300c	31.257b	13.563b	14.356a	43.686a
		(51901)	(0.46)	(2.21)	(0.98)	(0.69)
	2	155600a	18.493a	7.813a	13.380a	58.385a
		(19632)	(0.53)	(1.37)	(1.39)	(3.55)
	4	471650b	25.470b	8.997a	21.735a	51.722a
Agricultural fertilizers		(95147)	(0.93)	(1.90)	(1.54)	(8.04)
medium	6	736850c	22.731b	8.739a	12.060a	53.588a
		(149437)	(0.81)	(1.59)	(2.16)	(4.91)
		1065750d	27.632b	14.824b	21.913a	39.414a
	8	(18879)	(0.51)	(1.78)	(3.05)	(9.71)

The standard deviation is included in parenthesis.

The different letters on the side of the quantities indicate significant differences (two-way Anova and Tuckey's *a posteriori* test, $\alpha = 0.05$): $a < b < c$.

TABLE 3.

Average proximal composition (expressed in percentage) based on the dry weight organic of *Nitzschia thermalis var. minor* cultured in 18-l plastic containers with "f/2" culture media (Guillard & Ryther, 1975) and with agricultural fertilizers.

Culture medium	Day	Cell density	% Proteins	% Carbohydrates	% Lipids	% Ash
	2	65468a	*	23.923b	*	52.537a
		(11782)		(2.75)		(3.03)
	4	83125a	33.505b	12.110a	21.374a	48.205a
		(12311)	(0.90)	(1.35)	(1.12)	(0.28)
"f/2" Medium	6	88750a	25.473a	8.679a	39.808b	33.731a
		(16250)	(0.98)	(0.74)	(1.66)	(4.09)
	8	73750a	25.497a	7.255a	41.201b	34.788a
		(13994)	(0.99)	(0.61)	(1.82)	(2.87)
	10	69674a	29.697a	8.321a	58.601c	38.192a
		(12521)	(3.68)	(0.38)	(8.41)	(6.11)
	2	34843a	35.579b	11.453a	24.370a	50.150c
		(15732)	(4.96)	(1.86)	(2.44)	(1.15)
	4	77812a	28.930a	12.831a	18.973a	44.427a
		(7739)	(0.55)	(1.87)	(1.72)	(2.23)
Agricultural fertilizers	6	63112a	29.700a	10.695a	34.247b	34.875b
medium		(15909)	(1.43)	(1.54)	(2.22)	(5.98)
	8	94687b	26.557a	9.037a	41.340b	31.041b
		(34248)	(4.08)	(0.95)	(0.67)	(3.12)
	10	88906b	26.404a	8.875a	44.549b	31.993b
		(1767)	(1.00)	(0.57)	(3.12)	(5.08)

*Not measured

The standard deviation is included in parenthesis.

The different letters on the side of the quantities indicate significant differences (two-way Anova and Tuckey's *a posteriori* test, $\alpha = 0.05$): a < b < c.

TABLE 4.

Average proximal composition (expressed in percentage) based on the dry weight organic of *Navicula incerta* cultured in 18-l plastic containers with "f/2" culture media (Guillard & Ryther 1975) and with agricultural fertilizers.

Culture medium	Day	Cell density	% Proteins	% Carbohydrates	% Lipids	% Ash
	2	97150a	35.153a	7.182a	9.242a	39.997a
		(43838)	(0.98)	(1.09)	(1.16)	(3.93)
	4	45965b	35.405a	11.129a	13.288a	31.089a
		(37825)	(4.31)	(1.45)	(0.62)	(10.85)
"f/2" Medium	6	464262c	30.518a	11.021a	14.289a	31.835a
		(6884)	(5.16)	(0.81)	(1.30)	(5.96)
	8	530287d	30.973a	12.082a	15.161a	31.447a
		(68634)	(1.27)	(2.03)	(1.02)	(5.58)
	10	535625d	26.336a	13.693a	13.783a	28.007a
		(88838)	(7.24)	(1.84)	(0.72)	(4.17)
	2	62150a	33.530a	7.589a	9.820a	39.813a
		(22899)	(6.03)	(1.18)	(1.13)	(7.06)
	4	263100b	30.159a	13.193b	12.205a	40.727a
		(34791)	(2.14)	(0.77)	(1.42)	(11.62)
Agricultural fertilizers	6	433725c				
medium		(106521)	*	*	*	*
	8	525937c	25.618a	10.838b	13.034a	41.107a
		(80247)	(2.15)	(0.31)	(0.95)	(0.63)
	10	345387b	23.344a	12.886b	10.692a	35.636a
		(17677)	(1.36)	(0.74)	(0.58)	(3.97)

* Not measured

The standard deviation is included in parenthesis.

The different letters on the side of the quantities indicate significant differences (two-way Anova and Tuckey's *a posteriori* test, $\alpha = 0.05$): a < b < c < d.

(Table 4). There were no significant differences between both treatments ($F = 0.082$, $P = 0.777$).

The highest carbohydrate percentage was obtained on the last culturing day in the control medium, whereas in the experimental medium, the higher value appeared on the fourth culturing day (13.193). There were no statistical differences between both treatments (Table 4) ($F = 2.965$, $P = 0.097$).

The lipid percentage both in the control and experimental media showed the highest values on the eight culturing day (15.161 and 13.034 respectively). There were no significant differences in lipid percentage between both treatments ($F = 0.225$, $P = 0.639$).

The organic dry weight and ash content for *Navicula incerta* were similar to those of the other two species. However, for this diatom, protein, carbohydrate, and lipid concentrations were around two or three times higher than those of the other two species, especially at the end of the culture irrespective of treatments (Table 4).

DISCUSSION

Some of the most important factors that must be considered for the production of microalgae biomass are quality and availability of nutrients, quantity and quality of light, pH, and temperature. All these variables can modify or limit growth rate, biomass production and the chemical composition of the microalgae and, as a result, their nutritional value (Kawamura & Hirano 1992, Flores-Vergara 1998).

The results obtained in this study showed that the chemical composition of each benthic diatom species used was similar irrespective of whether it was cultured in the experimental or control "f/2" medium. However, from the three strains evaluated *Navicula incerta* showed the highest values on protein, organic dry weight and ash content, as well as some important differences in chemical constituent. The weights evaluated showed that this strain is heavier and bigger than the other two benthic diatoms used in this work; supporting López-Elias and Voltolina (1993) and Perera-Carbonell (1994) who worked on planktonic microalgae. These authors did not find differences in the growth rate and chemical composition of microalgae reared in a traditional culture medium or one with agricultural-type fertilizers. Gracida-Valdepeña (1999) found some differences in the microalgae quality, but not in their quantity when cultured with agricultural fertilizers rather than in the "f/2" medium.

The chemical composition of our three diatoms strains was similar to those of other benthic diatoms (e.g. *Amphora catenula, Navicula cincta, Nitzschia closterium* and three other strains of *Nitzschia* sp.) cultured in medium "f" described by Guillard and Ryther (1962). However, these cultures were maintained under different light intensity (80 and 160 $\mu Em^{-2}\ s^{-1}$) and temperatures (15, 20 and 25 °C). Flores-Vergara (1998) reports values of proteins from 20 to 40%, carbohydrates from 4 to 25% and lipids between 5 and 45%.

The high ash content resulting in the benthic diatoms cultured could have originated from satisfied requirements for silica for frustule formation, as this trend was similar in both culture media. Several diatoms may be capable of continuous uptake and storage of silicon in their vacuoles (Werner 1977). Studies on planktonic diatoms *Thalassiosira pseudonana* and *Chaetoceros calcitrans* indicated that ash content can vary between 27 and 35%, and that low ash percentage in phytoplankton implied low levels of intracellular potassium and sodium (Whyte 1987).

The structural strength of diatom cells may depend on factors such as culture conditions and diatom growth phase (Kawamura & Hirano 1992). This strength could affect the ability of the abalone postlarvae to digest these diatoms (Roberts et al. 1999), as when the diatom cells are weakly silicified, the abalone radula appears to deform the cell easily. Since physical rupturing of diatom cells probably relies solely on the radula or other bucal apparatus (Roberts et al. 1999), if the postlarvae cannot break the cells of the diatom strain, it cannot absorb the diatom's cell contents (Kawamura 1996).

No previous work has considered the use of agricultural fertilizer to grow benthic diatoms, although they are often used as sources of nitrogen and phosphorous on several Mexican abalone farms. However, no previous evaluation of quantity or quality of microalgal biomass production using this method has been made. The use of fertilizers on Mexican farms reduces production costs. There, commercial "f/2" medium is used exclusively for inoculum, while culture medium, prepared with fertilizers, is used frequently in the massive production.

In this work, the experimental culture medium contained three nitrogen forms: ammonia, nitrate and urea. Physiological studies on phytoplankton cultures showed that assimilation of the reduced form, ammonium, is preferred and inhibits the nitrate reductase production in the microalgal cell and provides lower energetic cost (Wheeler 1983). Usually higher concentrations from 0.5 to 0.1 $\mu mol\ l^{-1}$ inhibits the nitrate uptake (Syrett 1981). These reasons could explain the good chemical composition of our three benthic diatoms strains cultured on the experimental medium. However, no previous studies have been found concerning the production of benthic diatoms using fertilizers as a culture medium, although this is very common in planktonic algae (e.g. *Thalassiosira pseudonana*) (Dortch et al. 1991).

The lower production costs achieved when using the alternative medium, compared to when using the control culture medium, is a great advantage. We found that it was eight times lower than when the control culture was used (Table 1). Many researchers have emphasized this fact. For example, McAnally-Salas et al. (1992) established a 98% saving by formulating a cultured medium from agricultural fertilizers, compared to the cost of the "f/2" culture medium. Valenzuela-Espinoza (1997) reports that the agricultural fertilizer medium is up to eight times less expensive than the "f/2" culture medium. This alternative medium could be successfully used in abalone farms, where large quantities of microalgal biomass with adequate chemical composition is required at a low cost. Liquid fertilizers appear potentially useful for preparation of culture media. The advantage of these fertilizers is their high solubility compared with those of solid fertilizers (González-Rodríguez & Maestrini 1984, Simental-Trinidad 1999). Finally, we recommend the utilization of the studied agricultural fertilizers for the culture of benthic diatoms, since the biochemical composition of the cells was similar to those obtained with the culture medium "f/2".

CONCLUSIONS

The utilization of agricultural fertilizers in mass culture of benthic microalgae is possible because they have a biochemical composition and growth rate similar to those obtained with the control culture medium "f/2" and production costs are substantially reduced.

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ECOLOGY AND POST-SETTLEMENT SURVIVAL OF THE EZO ABALONE, *HALIOTIS DISCUS HANNAI*, ON MIYAGI COASTS, JAPAN

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ABSTRACT Settlement and survival of the ezo abalone, *Haliotis discus hannai* were examined in near-shore habitats at exposed to sheltered sites on Miyagi coasts, Japan from 1996–1999. Larvae settled mainly in the boundary zone at 2–7 m depth according to site between shallower *Eisenia* forests and deeper crustose coralline flats at densities of ~200–800/m^2. Growth rates were 30–40 μm/day. Instantaneous natural mortality rates, M, in the first 55 days after settlement were 3.7–5.5/month. Sampling of the habitat during the week after settlement showed a 29–37% incidence of atrophied post-larvae of ~500 μm shell length indicative of starvation. Laboratory experiments suggested that post-larvae exhaust the natural food supply on crustose coralline algae in the first week after settlement and begin to starve at a size of ~450–500 μm in the absence of a suitable diatom diet. One-year-old juveniles were mainly found in the boundary zone but with increasing age juveniles moved into the shallower *Eisenia* forest where food algae is abundant.

KEY WORDS: settlement, recruitment, survival, density-dependent mortality, crustose coralline algae, isoyake, *Haliotis discus hannai*

INTRODUCTION

An understanding of the ecology and dynamics of recruitment in abalone populations is crucial to management of their fisheries (McShane 1992, McShane 1996), especially when those fisheries are in decline through overfishing. Numerous studies have shown the importance of crustose coralline algae (CCA) as a preferred substratum for larval settlement of abalone (Saito 1981, Shepherd & Turner 1985) but few studies have examined the survival of settlers (Shepherd & Daume 1996) or the causes of mortality.

Two hypotheses, predation and food availability, have been proposed to explain the early high mortality (M) of post-larval abalone. Morse et al. (1979) and Naylor and McShane (1997) suggested that infaunal polychaete predators controlled the abundance of abalone post-larvae and McShane (1991) thought that bulldozing by urchins may also inflict high M. A corollary of this hypothesis is that the characteristics of the CCA substratum may be critical, either by providing a protective micro-habitat, e.g. in the lumpy growth-form of certain CCA (Shepherd & Daume 1996), or conversely by favoring loss from wave-shear on smooth forms of CCA (McShane 1996).

Alternatively, food availability has been considered to play a key role in the growth and survival of post-larvae (Kawamura et al. 1998). While growth and survival on a CCA substratum is superior to those on any other substratum in the first few days to a week after settlement (Morse 1984, Hooker & Morse 1985, Daume et al. 1999a), thereafter the quality, type and abundance of diatoms become critical for growth and survival. Both factors, predation and food availability, together were considered by Shepherd et al. (2000) to be the main determinants of the carrying capacity of the CCA habitat.

Our earlier study on the spawning, larval dispersal and settlement of *Haliotis discus hannai* Ino on Miyagi coasts (Sasaki & Shepherd 1995) showed that spawning was epidemic and induced by typhoons. This enabled the prediction of spawning events from the occurrence of typhoons and the estimation of the time of larval settlement. We monitored larval settlement of *H. d. hannai* following annual typhoons from 1995 to 1999 in Samenoura Bay. Specifically we asked what the characteristics were of the habitat in which larvae of this abalone settled, in what density did they settle and were post-settlement processes limiting recruitment to wild populations. In this article we describe the preferred zone of settlement within the near-shore rocky reef ecosystem, post-larval survival and subsequent movement and habitat of juveniles to age five years. We also carried out ancillary experiments to elucidate the cause of post-settlement mortality.

MATERIALS AND METHODS

Description of Study Sites

The coast of Samenoura Bay in northeast Honshu Island where the study was done (Fig. 1) ranges from sheltered to exposed. Following the description of Taniguchi (1991) we recognize three distinct sublittoral zones according to algal dominants, named A–C in this article, on rocky bottom.

Zone A is an *Eisenia bicyclis* forest going from low water to a depth of 2–4 m. The zone is shallowest in sheltered conditions and its depth increases with increasing exposure to wave action. *Sargassum yezoense* is a sub-dominant at the shallowest depths.

Zone B is a boundary transition zone between the *Eisenia* forest and the deeper CCA zone C, and is characterized by *Acrosorium polyneurum*, and, to a less extent, by *Chondrus ocellatus* and *Dilophus okamurai*.

Zone C, called the CCA flats (in Japanese isoyake), comprises boulders with high CCA cover but bare of erect algae and grazed intensively by the sea-urchin *Strongylocentrotus nudus*. We have no data on the species of CCA present, but in southern Hokkaido the main species are (with mean percentage cover in brackets): *Lithophyllum yessoense* (69%), *L. okamurai* (5%), *Lithothamnium japonicum* (8%) and *Neogoniolithon* sp. (6%) (Noro et al. 1983).

The distribution of common algal dominants (Taniguchi 1991) in the three zones is shown in Figure 2 and illustrated in Figure 3.

Post-Larval Sampling

We chose three sampling sites, numbered 1–3, to provide a range of exposures to wave action (Fig. 1). The three zones and the same algal flora were present at each site, but the zones differed in

*Corresponding author.

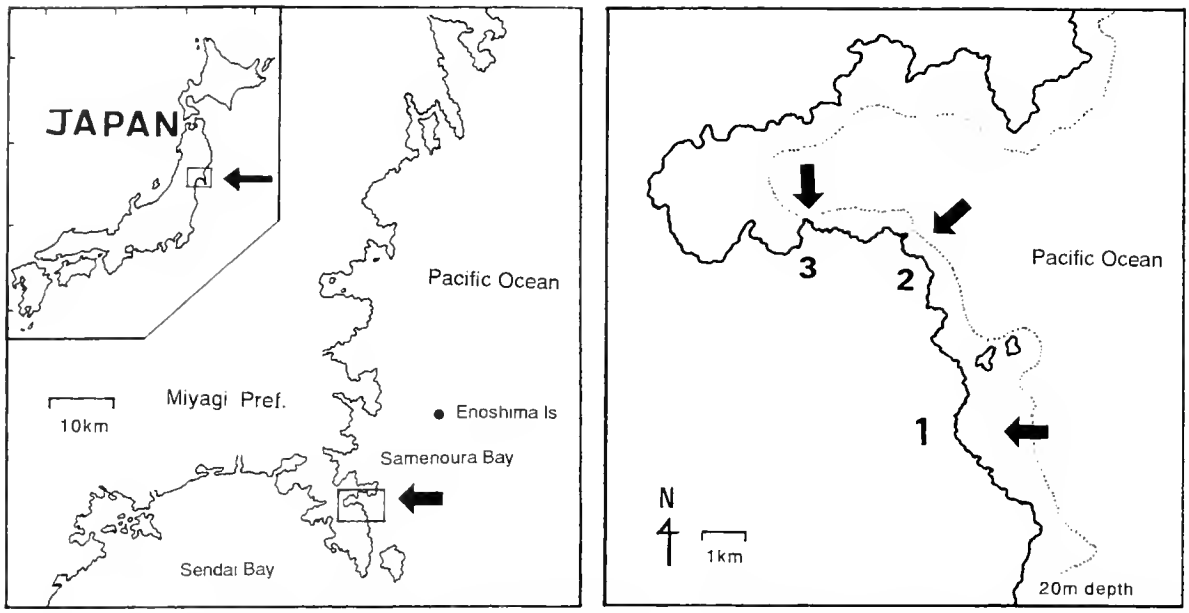

Figure 1. The coast of Miyagi Prefecture (left) and the study sites in Samenoura Bay (right) in northeast Honshu island, Japan (inset).

horizontal extent. Site 1 is the most exposed, facing the Pacific Ocean, with a gentle slope of 4/100, Site 2 at the entrance of Samenoura Bay is moderately exposed with a bottom slope of 6/100 and Site 3 within the Bay has a slope of 6/100 (see Figs. 3, 4, 5 for the horizontal extent of the respective zones).

At each site we estimated the abundance of newly settled abalone spat by taking, with the aid of SCUBA, 4–6 boulders, each 20–30 cm diameter, per sample at regular sampling intervals along a transect normal to the coast and intersecting each of the three zones. The boulders were taken to the laboratory and immersed in 0.4% formaldehyde solution; then the spat was washed off the substratum with a water jet, examined under a binocular microscope, and individual shell lengths measured. We also recorded those spat showing atrophy (i.e. shrinkage) of the soft tissue within the shell. The length, width and depth of each boulder were measured and the total area of the sides and top of a rectangular box of those dimensions was taken as an estimate of the area of the boulders. Densities were converted to numbers/m^2.

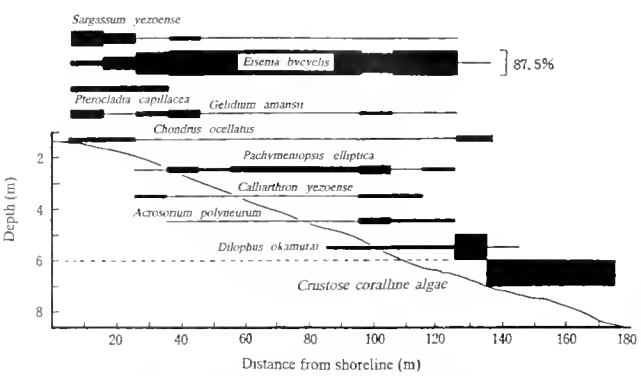

Figure 2. Schematic horizontal distribution with depth of major benthic algae at Site 1 (after Taniguchi 1991). The sloping line represents rocky bottom, the thickness of the distribution lines indicates % cover and the dashed line at 6 m depth is the boundary between Zones B and C.

Sampling of boulders for newly settled spat was done annually for 2 y at Site 1, for 3 y at Site 2, and for 2 y at Site 3. In 1997, boulder sampling at Site 1was repeated four times over 55 days and the natural mortality rate of spat estimated by regressing the natural logarithm of density versus time.

At Site 1 we also deployed strings of 80 scallop shells anchored just above the bottom as artificial collectors at regularly spaced intervals to independently monitor spat settlement. The surface area of the shells was estimated and the results presented as numbers/m^2.

Post-Larval Survival Experiments

Two experiments, one using dried boulders in the field, and the other with cobbles covered with living CCA in aquaria, were carried out to measure post-larval survival in different conditions. In the former experiment we placed sun-dried boulders in Zone B at Site 1 and compared the number and size of post-larvae on dead substrata and nearby natural control substrata. We placed the boulders in the field on 22 September 1997 on the same day that settlement began, and retrieved them four days later.

In the second experiment (called the aquarium experiment) 2,500 *H. d. hannai* larvae were released into each of five 10-L aquaria with living CCA cobbles of ~10 cm diameter set on the bottom and their survival monitored over 14 days. The cobbles had been previously brushed and washed in filtered sea-water to remove predators and microalgae including diatoms. The aquarium water temperature was 18–20°C. The contents of the aquaria were preserved in formalin successively at two- to three-day intervals and the empty shells and those living to that time measured and counted. The aquarium experiment was repeated twice with the same protocols.

Juvenile Sampling

The abundance of juvenile (one to five y) abalone was estimated at each site in January 1998 by searching within each zone within a 5 × 5 m quadrat with three replicates. All such surveys were done by one of us (RS) to standardize searching

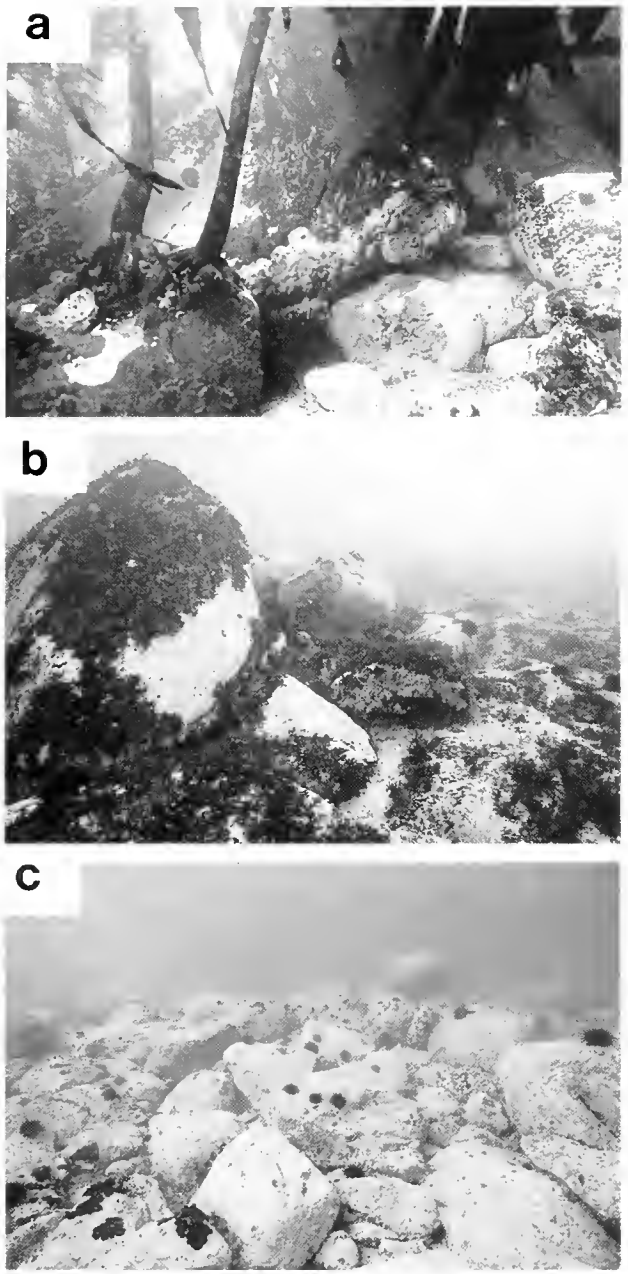

Figure 3. Photographs of the bottom at Site 1 at the entrance to Samenoura Bay. a: Zone A showing canopy of *Eisenia bicyclis*; b: boundary Zone B showing low cover of the macroalgae, *Acrosorium polyneurum* and *Dilophus okamurai*, and c: Zone C showing sea-urchin *Strongyloceutrotus nudus* in isoyake area.

efficiency. Small abalone were aged by the annual growth check which is conspicuous in this species (Sakai 1960).

RESULTS

Distribution and Density of Post-Larvae

The distribution of density of post-larvae along transect lines in relation to depth and habitat is shown in Figures 4–6.

At Site 1 (Fig. 4) settlement was monitored on scallop-shell collectors and showed a maximum density at a distance of 160 m from shore at 5 m depth in the boundary zone (B). Mean post-larval shell length was 415 μm (SE 60 μm) five days after settlement. In 1996 post-larval density ranged from 0–30/m^2 in Zone A to 220/m^2 in Zone B with intermediate values in Zone C. Mean shell length was 425 μm (SE 75 μm). In 1997 with increased sampling intensity at 15 m intervals there is a clear modal density of 340 spat/m^2 at a distance of 180 m from shore at the outer edge of Zone B. Mean shell length was 510 μm (SE 40 μm).

At Site 2 (Fig. 5) there was a peak in spat density in Zone B in 1997 (mean shell length 340 μm, SE 20 μm), nearly uniform spat density in 1998 in all zones to a depth of 6 m (mean shell length 910 μm, SE 830 μm), and in 1999 a modal density at 80 m from shore in Zone C (shell length 380 μm, SE 45 μm).

At Site 3 (Fig. 6) there was a weak settlement in 1998 with a peak density in Zone C (mean shell length 695 μm, SE 575 μm), and a stronger settlement in 1999 with a peak density at the inner edge of Zone C (mean shell length 370 μm, SE 50 μm).

In summary the results show trends of declining spat density with increasing shelter and peak densities at the boundary between Zones B and C with lower densities further in- and off-shore at each site.

Post-Larval Growth and Mortality

Length-frequency data at Site 1 after the 1997 settlement (Fig. 7), shows modes at 390 μm and 410 μm on 25 September increasing to 490 μm and 560 μm respectively 6 days later, and finally a single apparent mode at 1–2 mm 13 days after that. In contrast, the "dead" boulders showed a single peak at 290 μm early after settlement. The length-frequency distribution of spat on "dead" boulders differed significantly from that on natural boulders obtained in the same period ($\chi^2 = 42.2$; $P < 0.001$), but the mean size of spat did not differ significantly between the two sets of boulders ($t = 1.6$; $P = 0.1$) although mean size was 10 μm smaller on the dead boulders.

The density of post-larvae at Site 1 sampled up to day 55 after putative settlement declined exponentially (Fig. 8). The estimated instantaneous monthly mortality rate, M, for the first 27 days was 5.3 (SE 0.7) and for the whole 55 days was 3.7 (SE 0.6). Length-frequencies of post-larval samples obtained at Site 1 after the 1996 and 1997 settlements (Fig. 9) showed that the mean length of spat with atrophied foot was 504 μm (SE 78 μm) and that of normal spat was 387 μm (SE 56 μm) in 1996, and 508 μm (SE 44 μm) and 512 μm (SE 41 μm) respectively in 1997. The spat with atrophied foot comprised 37% of the 1996 sample (N = 92) and 29% of the 1997 sample (N = 304).

In the first aquarium experiment 98% of the released larvae settled on the cobbles and survived to day 5, but declined sharply and more or less linearly after day 7 to 53% on day 14 (Fig. 10). Mean shell length increased at a daily growth rate of ~40 μm to a mean length of 478 μm (SE 13 μm) and then practically ceased, reaching a mean length of 499 μm (SE 22 μm) at day 14 when the experiment ended.

The mean shell length of spat that died was 435 μm at five days and increased only slightly after that; the mean shell length of "dead" shells was 468 μm (SE 19 μm) for the whole 14 days. In the second experiment, survival was 85% after 14 days and in the third experiment 27% after 16 days; in both the last two experiments survival was 99% for the first week and declined steeply after that.

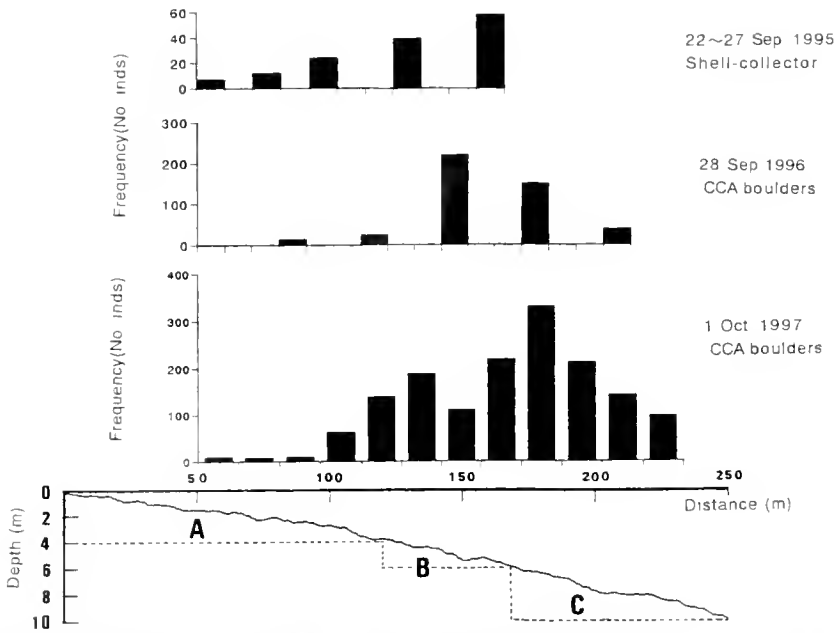

Figure 4. Vertical distribution of density of *Haliotis* post-larvae in numbers/m^2 along a transect normal to the coast at Site 1 in 1995, 1996 and 1997, with depth profile of the bottom and horizontal extent of Zones A, B and C.

Distribution of Juveniles

Length-frequency distributions of juvenile abalone in Zones A, B, and C for all sites combined (Fig. 11) show that the largest mean size and the highest densities of juveniles were in the *Eisenia* forest (A) and the lowest densities were in the CCA flats (C). The mean density of juveniles was 1.06/m^2 in Zone A, 0.83/m^2 in Zone B and 0.27/m^2 in Zone C. Mean densities in Zones A and B did not differ significantly (t = 1.3; NS) but did differ significantly between Zones B and C (t = 3.1; P, 0.01). Size distributions in Zones A and C differed significantly from those in Zone B (for AB and BC comparisons χ^2 = 119 and 35 respectively: $P < 0.001$). Mean shell lengths in the three zones were 79 mm, 46 mm and 62 mm respectively. The age distribution of juveniles in the three zones combined (Fig. 12) shows that the density of the 0+ and 1+ years classes was 6–7 times higher in Zone B than in the other zones. The 0+ and 1+ year classes combined comprised 6% of all abalone on Zone A, 49% in Zone B and 25% in Zone C. The age distributions in Zones A and C also differed significantly from that in Zone B (for AB and BC comparisons χ^2 = 486 and 123 respectively; $P < 0.001$).

A change in the ratio of the density of each year class in Zone A to the density of the same year class in Zone B (Fig. 13) indicates the likely direction of migration between the two zones assuming that there is no differential M between zones. The low ratios for the 0+ and 1+ year classes shows high settlement and retention of those year classes within the zone, and the sigmoid curve for older year-classes suggests strong initial migration of the 2+ year-class from Zone B to A levelling off in the older year-classes.

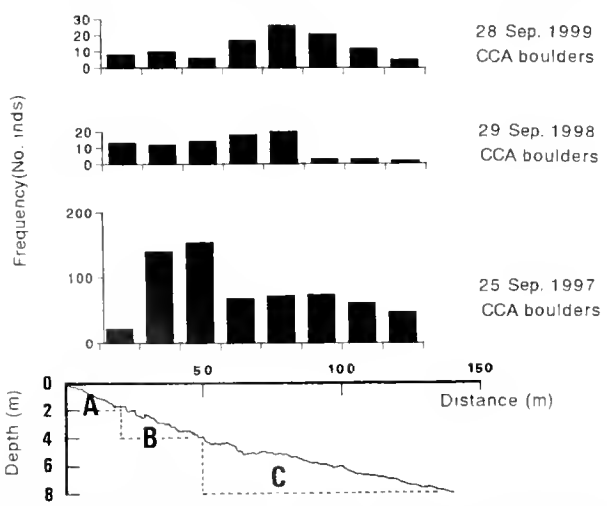

Figure 5. Vertical distribution of density of *Haliotis* post-larvae in numbers/m^2 along a transect normal to the coast at Site 2 in 1997, 1998 and 1999 with depth profile and zones as in Figure 4.

DISCUSSION

Post-Larval Distribution

This paper provides the first published account of the dynamics of settlement of *H. d. hannai*, survival of spat, and subsequent

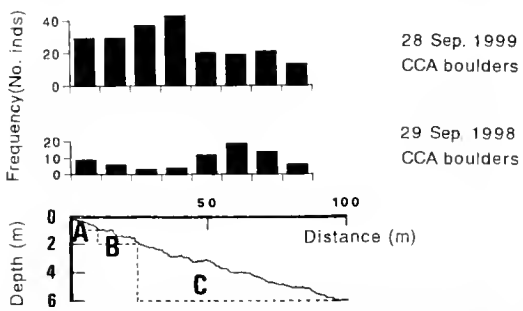

Figure 6. Vertical distribution of density of *Haliotis* post-larvae along a transect normal to the coast at Site 3 in 1998 and 1999 with depth profile and zones as in Figure 4.

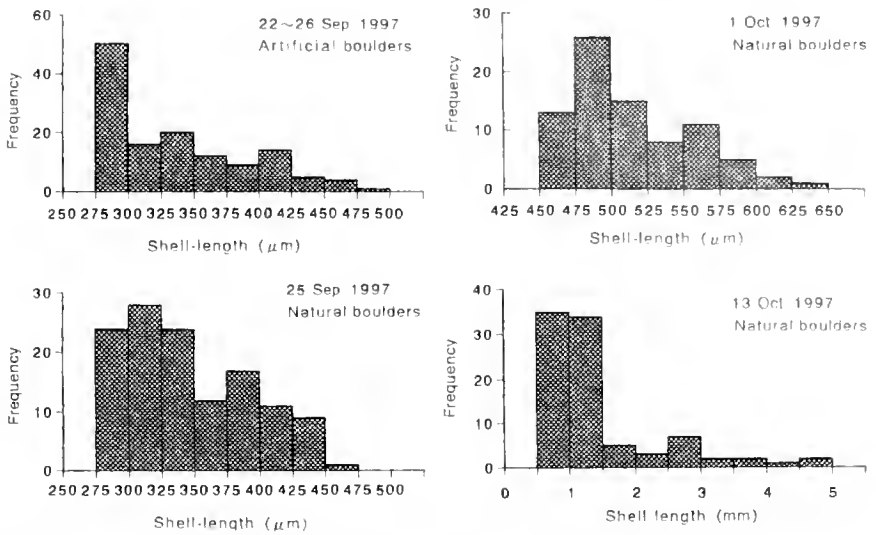

Figure 7. Length-frequency distributions of *Haliotis* post-larvae on dried and natural boulders at successive time periods after settlement on about 22 September 1997 at Site 1.

movement of juveniles in relation to the near-shore habitats in north-east Honshu. Our earlier study (Sasaki & Shepherd 1995) examined larval dispersal in Kesennum and Samenoura Bays, and the vertical distribution of larval settlement in Kesennuma Bay. Larvae were advected shoreward by wind-driven on-shore currents and the highest density of settlers occurred on high energy coasts at 4–6 m depth. Larval densities declined sharply with increasing shelter within bays. Sasaki and Shepherd (1995) suggested that the preferred depth of settlement was fixed during the late veliger stage when highest larval concentrations were also found at 4–6 m depth.

The settlement of this abalone in Samenoura Bay (this study) follows the same pattern as in Kesennuma Bay, 120 km north (Sasaki & Shepherd 1995). The *Eisenia* forest habitat (Zone A) and adjacent habitats (B and C) are widest on exposed Pacific coasts and each zone narrows sharply with increasing shelter. The intensity of settlement is correlated both with the distribution of late veligers spatially and vertically in the water column (Sasaki & Shepherd 1995), and with the spatial extent of the *Eisenia* forest zone, which provides the source of algal food for this abalone (Taniguchi 1991). But why is the boundary zone the apparent preferred habitat for settlement of this abalone?

The other main herbivores in abalone habitat at our study sites are *Tegula lischkei, T. pfeifferi,* and *T. rustica* which all settle preferentially in Zone B and subsequently move into the *Eisenia* forest like *H. d. hannai* (R. Sasaki unpublished data). These *Tegula* species, as yet indistinguishable as post-larvae, settle at about double the density of *H. d. hannai* and may be competitors for diatoms preferred by the abalone. The habitat of *S. nudus* is coralline flats (Zone C). Further, the environmental cues which trigger epidemic spawning in *H. d. hannai* also trigger spawning of *Tegula* spp. and the sea-urchin *Strongylocentrotus nudus* (Sasaki & Shepherd 1995). We hypothesize that the CCA substratum is optimal for all four molluscs in the boundary zone but becomes less suitable with increasing depth. Seki (1997) found slow growth rates and high M of post-larval *H. discus hannai* in CCA habitat. CCA morphology varies according to the presence of an algal canopy, near-shore hydrodynamics and with depth (McShane 1996) and its suitability as a substratum for abalone post-larvae

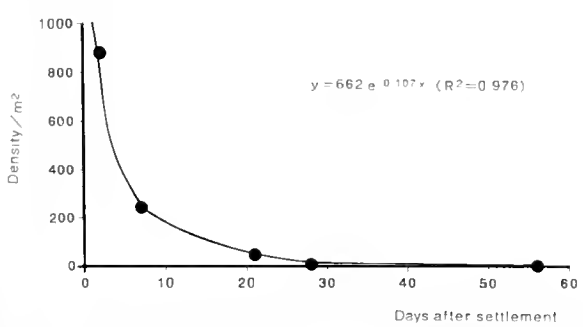

Figure 8. A plot of change in density in numbers/m² of surviving *Haliotis* post-larvae collected from crustose coralline boulders in Zone B at Site 1 in 1997.

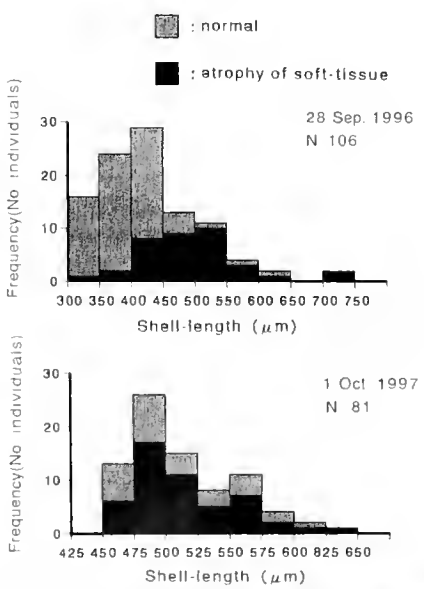

Figure 9. Length-frequency distributions of *Haliotis* post-larvae in 1996 and 1997. Shells with normal and atrophied soft tissue are distinctively shaded.

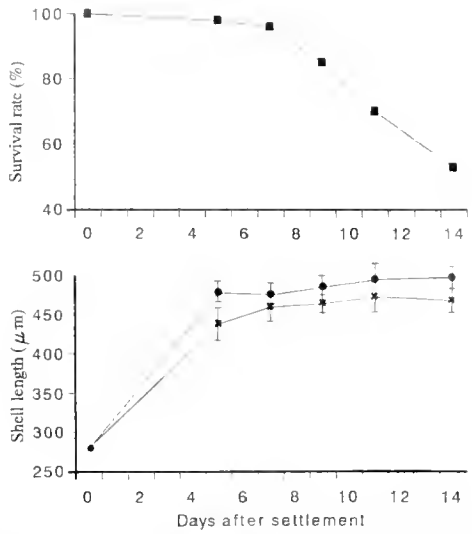

Figure 10. (upper) Survival (%) of *Haliotis* post-larvae on CCA-covered cobbles from which diatoms had been removed in an aquarium over 14 days. (lower) Growth of *Haliotis* post-larvae (circles) and size of dead shells (crosses) over time. Bars are standard errors.

also varies according to these factors and other local contingencies such as presence and abundance of predators. The dominant polychaete in CCA at our study sites is the detritus-feeding cirratulid *Dodecaceria concharum* (Hayashi et al. 1982) and we have no

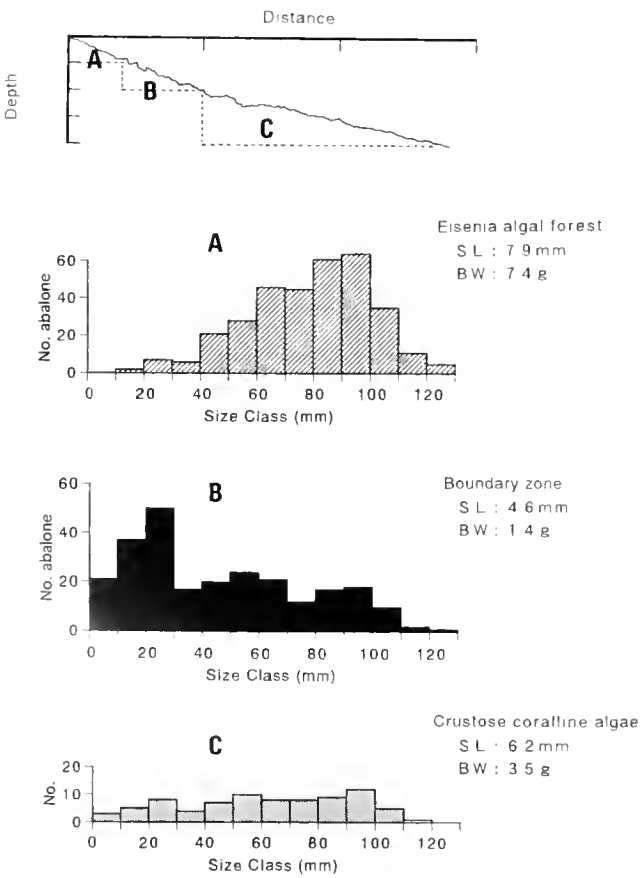

Figure 11. Length-frequency distributions of abalone for all sites combined for each of the three zones, A, B and C sampled in January 1998. SL = mean shell length and BW = mean in-shell weight.

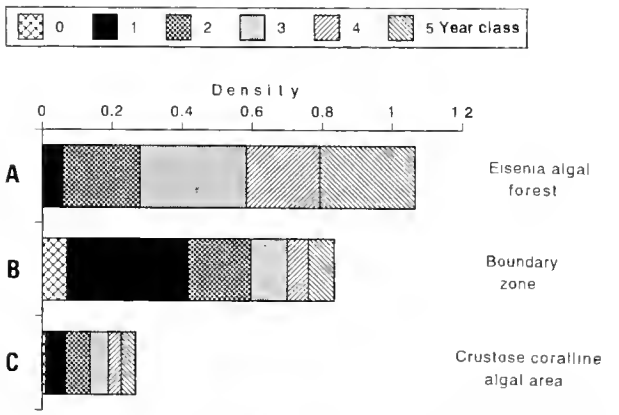

Figure 12. Mean density of five age classes of *H. d. hannai* in the three zones in 1998.

evidence that they consume abalone spat as in McShane's experiments.

Survival

Our M values of 3.7–5.3/month according to the period examined are higher than our earlier values in Kesennuma Bay (1.4–2.7/month), but initial densities were ~900/m^2 compared with only ~200/m^2 in the former study. Shepherd (1998) and Shepherd et al. (2000) reviewed earlier studies on post-larval M and showed strong density-dependent M in the range 0.6–2.7/month; our present study is consistent with this hypothesis. But what is the mechanism for density-dependent M?

Our data suggest that starvation is a major contributing factor to the high M at our study site, and is how we summarize the evidence. First, the very high growth rates and low M on CCA cobbles show that CCA is the optimal substratum for settling post-larvae for the first five to seven days after settlement. Experiments by Daume et al. (1999a, 1999b) also favor this conclusion. The cessation of growth at ~450–500 μm thereafter suggest that food became limited and induced the increasing mortality that occurred from day eight to day 14. This is a critical phase in the life of a post-larval abalone, and diatoms are essential for further growth and survival (Kawamura et al. 1998, Daume et al. 2000). The highly skewed distribution of length-frequency data on dried boulders in the field experiment (Fig. 7) suggest that relatively few larvae grew beyond the size at settlement or, alternatively, that M of larger post-larvae was higher in comparison with natural boulders.

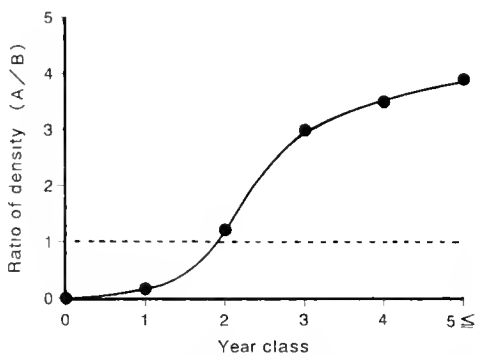

Figure 13. Plot of the ratio of density of each age class in the *Eisenia* forest (A) to the density of the same age class in the boundary zone (B).

Lastly, the apperance of atrophied post-larvae at modal sizes of 475–550 μm (Fig. 9) suggests that the high densities of post-larvae exhausted the natural diatom supply soon after the critical size when diatoms dominate the diet of post-larvae (Kawamura et al. 1998). In independent experiments with plankton trap-nets set at Site 1 in October and in other years, we have also recorded atrophied post-larvae of mean length 740 μm (SE 240 μm) as well as other small gastropods presumably washed off the substratum by wave turbulence. This suggests that starving post-larvae are susceptible to mortality from wave-shear as proposed by McShane (1996). Shepherd et al. (2000), in reviewing the evidence for density-dependent M of post-larvae, suggested that in southern Australian habitats, the carrying capacity of CCA boulders was ~100 post-larvae/m^2. From the results of this study and our earlier one we hypothesize a carrying capacity of <200/m^2 in terms of food availability on Miyago coasts. It is also possible that *Tegula* spp. may compete as post-larvae with post-larval abalone and so further reduce the carrying capacity of the habitat for abalone.

Distribution of Juveniles

The pattern of distribution of juveniles is initially similar to that of post-larvae, although in practice we found that highest juvenile densities occurred within the boundary zone (B) but just shoreward of the area of highest density of post-larvae. Because the depth of the *Eisenia* forest decreases with increasing shelter, the band of highest juvenile density is shallower in more sheltered waters.

What is the adaptive significance of shoreward migration of this abalone? Ino (1966) and Nie (1992) stated that this abalone migrated into shallow water before spawning, implying some adaptive advantage such as increased larval dispersal. If so, this behavior is analogous to the movement of other abalone species into the swell or into increased current flow (Shepherd 1986, Clavier & Richard 1984). While this may be true at our study site, the obvious explanation is that *Eisenia* and associated algae are a preferred food (Taniguchi 1991, Shepherd & Steinberg 1992). Hence movement to this habitat must be advantageous for growth and reproduction. A similar size-related migration by the related species *H. kamtschatkana* from deeper water where larvae settle into shallower kelp habitat is also recorded in Alaska and Canada (Sloan and Breen 1988, S. A. Shepherd unpublished data). Such migration is quite different from the aggregation and movement of spawning adults onto the highest points of reefs in shallow water for spawning (Breen & Adkins 1980, Stekoll & Shirley 1993, unpublished observations).

CONCLUSIONS

Our study has important implications for the management of the declining *H. d. hannai* fishery in north-east Honshu. Abalone larvae advect shoreward according to local hydrodynamic conditions and settle at densities that depend on coastal topography and the extent of suitable habitat. Post-larval densities of both this abalone and *Tegula* spp. are highest in the boundary zone between the near-shore *Eisenia* forest and the deeper CCA habitat. However, post-larval survival appears to be food-limited because significant numbers of post-larvae five to seven days old show an atrophied condition. This raises the possibility that the abundance of *Tegula* post-larvae in the same habitat may affect the survival of post-larval abalone.

With increasing age juvenile abalone migrate into the *Eisenia* forest which provides an abundant food supply for adult abalone. To achieve an understanding of the causes underlying the decline of the fishery, studies should now focus (a) on the relation between recruitment strength of abalone into the fishery and the extent of the boundary zone, and (b) the competitive relations between abalone and *Tegula* post-larvae.

ACKNOWLEDGMENTS

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SUITABILITY OF AUSTRALIAN FORMULATED DIETS FOR AQUACULTURE OF THE TROPICAL ABALONE *HALIOTIS ASININA* LINNAEUS

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ABSTRACT The performance of *Haliotis asinina* Linnaeus fed four commercially available diets formulated for temperate Australian species was assessed relative to a natural diet of *Gracilaria edulis*. Parameters measured included growth in terms of total wet body weight (TWBW) and shell length (SL), survival, food conversion ratio (FCR), condition index (CI), and sexual maturation. The results of these measurements indicated that two of the formulated diets could be used for commercial culture of *H. asinina*. Nutritional and physical parameters of these diets were used in a multivariate regression analysis to identify variables that explained the most variation in growth between diets. Parameters that were selected by this analysis were the ratio of crude protein (CP) to gross energy (GE) and the rate that protein leached from the formulated diets. The resulting regression equation was highly significant in its explanation of the variation in growth ($P = 2.2 \times 10^{-6}$). To determine the quality of the dietary CP in each diet, the essential amino acid profile of each diet was compared to that of whole soft body tissue in *H. asinina*. Methionine seemed to be deficient in all of the diets. Animals fed *G. edulis* did not seem to suffer from a lack of n-3 PUFAs.

KEY WORDS: *Haliotis asinina*, formulated diet, growth, amino acid, fatty acid, sexual maturation, protein leaching

INTRODUCTION

Many abalone fisheries around the world are in decline (Hahn 1989a), and, as a result, research programs aimed at the efficient artificial production of a range of haliotid species are underway. One important aspect of such research is the identification of the nutritional requirements of commercially important abalone species (Fleming et al. 1996). This allows the formulation of cost-effective diets that provide a consistent and precise method of supplying the nutrients necessary for maximal growth. Diet formulations can also be easily modified as the nutritional requirements of an animal changes during development (e.g., postsettlement, growout, and broodstock diets).

Traditionally, the majority of abalone diet research around the world has focused on temperate species, because these form the majority of abalone exports worldwide (Hahn 1989a). However, recent research has indicated a growing demand for tropical abalone (Nateewathana & Hylleberg 1986, Singhagraiwan & Doi 1993, Jarayabhand & Paphavisit 1996). Jarayabhand and Paphavisit (1996) report that in Taiwan these smaller tropical species are preferred to the temperate species because of their size and delicate flavor.

The distribution, ecology, reproductive biology, and natural diet of the tropical abalone *Haliotis asinina* Linnaeus differ from Australian temperate species. *H. asinina* is found on intertidal coral reef flats throughout the Indo-Pacific (Gosliner et al. 1996); whereas, *Haliotis rubra* Leach and *Haliotis laevigata* Donovan are subtidal animals (Prince & Shepherd 1992) found on rocky temperate reefs. Populations of *H. asinina* on Heron Island (23°27′S; 151°55′E) spawn fortnightly in cue with lunar and tidal cycles from mid-October to mid-April (Counihan et al. 2001); whereas, temperate species in culture are artificially induced to spawn because of their relatively unpredictable and infrequent natural spawning patterns (Hahn 1989b). Temperate Australian abalone occur on reefs where macroalgae are relatively abundant (Dayton 1994); whereas, the abundance of frondose macroalgae on coral reefs is limited (Littler & Littler 1994). Sawatpeera et al. (1998) demonstrated that the gut contents of adult *H. asinina* collected from coral reefs of Samed Island, Thailand contained over 80% diatoms. These significant differences between temperate and tropical abalone suggest that diets formulated for temperate Australian abalone, *H. rubra* and *H. laevigata*, may not be suitable for *H. asinina*.

Research on tropical abalone nutrition is limited. Capinpin and Corre (1996) have investigated the growth rates produced by *H. asinina* fed a Japanese-formulated diet over 90 days, but performed minimal nutritional analyses on the diets used. Bautista-Tereul and Millamena (1999) also conducted a 90-day growth experiment and reported on the effects of dietary energy and protein on the growth of *H. asinina*, but not on the fatty acid (FA) profiles of the diets. These studies did not include a nutritional acclimatization period, and were run for only 90 days. Day and Fleming (1992) highlight the effects that previous diets may have on growth and recommend an initial period of nutritional acclimatization before any growth experiments, even for artificially produced animals that have a common diet history. Furthermore, they suggest that, in the case of *H. rubra*, a growth trial of less than 100 days will not be informative.

Here we report on the suitability of formulated diets that were produced for temperate Australian species (*H. rubra* and *H. laevigata*) for the culture of *H. asinina*. We determined: (1) the water stability of the diets; (2) rates of growth in terms of total wet body weight (TWBW) and shell length (SL) increase; (3) sexual maturation; (4) food conversion ratio; (5) dry feed intake; and (6) the condition index (CI) of the abalone after being fed these diets for 6 mo after a nutritional acclimatization period of 49 days. Growth rates promoted by the various diets are related to a range of nutritional parameters, including the crude protein (CP), total lipid (TL), gross energy (GE), fatty acid (FA), and essential amino acid contents of the diets fed.

MATERIALS AND METHODS

Diets

Samples of four formulated diets from two Australian abalone feed companies were obtained for comparison with a natural diet

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of the red seaweed, *Gracilaria edulis*. Animals were fed nightly to satiation, and uneaten food was removed the following morning. This procedure was done only once on weekends. *G. edulis* was chosen as the reference diet, because *Gracilaria* species have been reported to promote high growth in *H. asinina* (Singhagraiwan & Sasaki 1991, Singhagraiwan & Doi 1993, Capinpin & Corre 1996, Bautista-Teruel & Millamena 1999, Capinpin et al. 1999).

Animals and Management

Juvenile *H. asinina* used in the following experiments were derived from spontaneous spawnings by wild broodstock collected from Heron Island, Great Barrier Reef, Australia (23°27′S; 151°55′E). Abalone at the start of the experiment ranged in SL from 11.8 mm to 23.4 mm (average 18.3 ± 2.76 mm) and TWBW from 0.40 g to 2.75 g (average 1.32 ± 0.577 g). Individuals were blot dried, weighed, measured, tagged, and assigned to an experimental unit. Abalone were distributed among replicates so that there were no significant differences in SL or TWBW. Each diet treatment was replicated six times, and consisted of a 1.7-L lidded plastic container (21 × 14 × 7 cm) housing four abalone. A "spare" replicate of each diet treatment was also maintained. Animals in these spare replicates were used to replace any that died during the experiment. This maintained a constant density of animals per experimental unit for the duration of the experiment. Filtered (10 μm) seawater was heated to 28°C and delivered to individual containers at a rate such that the entire volume was exchanged once every 3 min. Seawater was not recirculated. Abalone were maintained in a 12L:12D light cycle for the duration of the experiment. Abalone were acclimatized to their respective diet treatments before the first TWBW and SL measurement for 49 days to reduce the residual effects of accumulated body stores from previous natural diets.

Growth Data Collection

SL and TWBW measurements of every animal were made at four weekly intervals. The trial was run for 24 wk. Growth promoted by each diet treatment was expressed as the increase in SL and TWBW over 24 weeks. All animals were examined for the presence of gonad tissue from the third month of the experiment onward. If gonad tissue was present, its color was recorded, allowing the sex of individual animals to be determined. As animals were individually tagged, a statistical comparison between male and female growth rates was also made.

Condition Index

A simple CI was used to evaluate the health of each animal at the end of the experiment using the equation:

$$CI = {}^{SFBW}/_{TWBW} \times 100$$

where TWBW is the total wet body weight (g), and SFBW is the shell free body weight (g). SFBW was estimated by subtracting the shell weight (SW) from the TWBW. Because the animals used in the feeding trial were to be used in other experiments, SW was estimated indirectly according to the equation:

$$SW = 3 \times 10^{-4} \times SL^{2.3245}$$

where SL equals shell length. This relationship had an R^2 value of 0.99 and was derived from over 300 shell measurements.

Food Conversion Ratio

The food conversion ratio (FCR) of animals reared on each diet was estimated during wk 18 to 20. Each night the wet weight of diet added to each experimental unit was weighed to within 0.1 mg. Twelve h later, uneaten feed was collected by passing the entire contents of each experimental unit carefully through a 1-mm^2 filter to remove feces, and then through preweighed Whatmann filter paper. The wet feed was then vacuum filtered and dried at 105°C overnight. For each experimental unit, the nightly dry feed intake (DFI) was calculated as follows:

$$DFI = f_1 \times {}^{DM}/_{100} - ({}^{f_2 \times 100}/_{WS})$$

where f_1 is the wet weight (g) of feed added each night, DM is the percentage of dry matter for each diet, f_2 is the weight (g) of uneaten dried feed, and WS is the water stability (%) of each diet. Water stability was calculated as the dry weight retained after immersion in seawater for 12 h under conditions identical to those at which the growth experiment was conducted. The nightly DFI over the 2-wk period was summed to give the total food intake (TFI) for each replicate. TWBW measurements were taken at the beginning and end of the 2-wk period, allowing the FCR to be calculated as follows:

$$FCR = {}^{WG}/_{TFI}$$

where WG is the TWBW gain (g) over the 2-wk period.

Gonad Index and Histology

During the final TWBW and SL measurements the gonad index (GI) of each animal was ranked following the method described by Singhagraiwan and Doi (1992). At the end of the growth experiment, the gonads from the two most mature animals of each sex on each diet were dissected, fixed in Bouin's fixative for 6 days, and washed three times in 70% ethanol over 3 days. Gonads were then embedded in paraffin, sectioned at 6 μm on a Spencer 800 microtome and stained with hematoxylin and eosin. Representative digital photos were taken with an Olympus DP10 digital camera attached to a BX60 microscope.

Nutritional Analyses

Representative samples of each diet were analyzed, usually in triplicate, for nutrient composition. The dry matter of each diet was determined by drying at 105°C for 12 h. Ash content was determined by incinerating dried samples at 200°C for 2 h, and then at 550°C for 12 h. Nitrogen content was analyzed by the Kjeldahl procedure, and CP was estimated by multiplying the nitrogen content by 6.25. TL was extracted with chloroform-methanol using the method of Folch (Folch et al. 1957). Saponification and esterification of the TL extract was performed following a modified method of van Wijngaarden (1967). Two replicate extracts of each diet were analyzed on a Hewlett Packard GC for FA content. GE was measured with an IKA Labortechnik Calorimeter.

For amino acid analysis, six wild adult abalone, ranging in TWBW from 63.6 to 149.3 g and in SL from 63 to 100 mm, were shucked, and the foot and viscera were blended together into a homogenate before being freeze dried. Two replicates of each diet were freeze dried and finely ground using a mortar and pestle. Samples were hydrolyzed in 6 M HCl at 105°C for 24 h. Free amino acids were derivitized using phenylisothiocyanate and separated using high-power liquid chromatography (HPLC) on a C-18 column with detection at 254 nm. Instrumentation was a Bio-Rad

2800 solvent delivery system with a Bio-Dimension UV/Vis monitor, controlled by a Series 800HPLC and v2.30.1a software. To compare diets directly, each amino acid was expressed as a proportion of the corresponding amino acid in *H. asinina* whole soft body tissue.

Protein Leaching

The rate of protein leaching from each diet was measured by the Bradford micromethod of protein estimation (Bradford 1976) using the Bio-Rad protein assay kit. 100 mL of 10 μm filtered seawater was placed into a clean conical flask and a background reading (before addition of any diet) was taken in triplicate. Approximately 1 g of each diet was weighed to within 0.1 mg and added to a flask. Triplicate optical density readings were taken at exactly 30 and 60 min after the addition of a diet treatment and thereafter hourly for a further 6 h using a Hitachi U-2000 spectrophotometer. Flasks were gently vortexed (approximately 120 cycles/min) in a Ratek orbital mixer at room temperature to simulate the effects of water flow and physical disturbance. A control consisting of 100 mL of seawater was run in parallel to the dietary treatments. All treatments were replicated three times, and optical density readings were related to a standard curve constructed from 10 concentrations of bovine serum albumin dissolved in seawater. The total protein leached (i.e., the protein concentration after 7 h) was expressed as a percentage of the total protein added to the flask (calculated from the protein content of each diet and the weight of feed added to each flask). It was found that *G. edulis* could not be included in this experiment, because it interfered with the dye-binding reaction of the Bradford method, resulting in protein concentrations less than the control. Therefore, to be included in the multiple regression analysis (see below) it was assumed that *G. edulis* leached no protein over a 7-h period.

Statistical Analyses

All statistical analyses were performed on STATISTICA for Macintosh (Statsoft 1994). All datasets were tested for violation of the assumptions of homogeneity of variance, normality, and independence of errors before further analysis. Datasets that failed any one of these assumptions were transformed and re-analyzed. The effect of tank was tested for all analyses where appropriate and was never found to be significant. Where significant differences between diet treatments were detected by one-way analysis of variance (ANOVA), a Student's–Newman-Keuls test (SNK) was performed for *a posteriori* comparison of treatment means. Growth data were analyzed by comparing the total increase in SL and TWBW between diets for the entire duration of the trial by one-way ANOVA. TWBW data were square root transformed before analysis to satisfy the assumptions of normality, independence of errors, and homogeneity of variance. SL data did not require transformation. The effect of diet and sex on GI was tested by a Kruskal- Wallis ANOVA by ranks. A forward stepwise multiple regression analysis was used to determine the nutritional parameters that explained the most variability in TWBW growth. Variables included in this analysis were: CP, TL, GE, the ratio of CP to GE, and the rate that protein leached from the diets (this rate was assumed to be zero for *G. edulis*, see above). Those variables that produced an F ratio in excess of 1.0 were included in the regression equation, and the tolerance level was set at 0.01. The significance level for all statistical analyses was set at $\alpha = 0.05$.

RESULTS

Water Stability and Nutritional Composition of Diets

The water stability and nutritional composition of four formulated diets and *G. edulis* were analyzed (Table 1). Diets were arbitrarily labeled 1 to 4. Diet 4 was significantly ($P < 0.05$) more water stable than all other formulated diets. When compared on a dry matter basis to *G. edulis*, formulated diets had significantly ($P < 0.05$) higher CP (21.8–40.0 vs 16.4%), TL (4.1–5.7 vs 0.8%) and GE (18.7–19.4 vs 9.0 MJ/kg) contents, respectively. The ash content and CP:GE ratio of diet 4 was significantly lower ($P < 0.05$) than all other diets (4.8–5.4 vs 3.3% for ash and 17.0–20.8 vs 11.7 mg/kJ for CP:GE). The rate of loss of protein from each of the formulated diets was essentially linear over 7 h, but seemed to begin to plateau by the final measurement, most noticeably in diet 4 (Fig. 1). The total loss of protein (expressed as mg dissolved protein per mL of leachate per gram of feed) over 7 h ranged from 9.1 to 18.0 (Fig. 1), with diet 4 losing the least protein and diet 3 the most. However, when the total amount of protein leached was expressed as a percentage of the total available protein (a more meaningful measure because of the different protein contents of each diet), diet 1 leached the lowest proportion of its protein content (9.6%), and diet 4 leached the highest proportion of its protein content (13.4%). These differences were significant between all diets, except diets 3 and 4 ($P < 0.05$; see Table 1).

Amino Acids

Relative to the formulated diets, the essential amino acid (EAA) composition of *G. edulis* protein contained higher amounts

TABLE 1.

Water stability and gross nutritional composition of four formulated diets and *Gracilaria edulis* fed to *Haliotis asinina*.

	Water Stability (%)	Protein Leached (mg/kg CP)	Moisture (g/kg)	Ash (g/kg)	CP (g/kg)	TL (g/kg)	GE (MJ/kg)	CP:GE (g/MJ)
Diet 1	76.6 (0.67)^{a}	96 (2.0)^{a}	111 (0.18)^{a}	54 (0.7)^{a}	400 (3.7)	45 (3.9)^{a}	19.2 (0.11)^{a,b}	20.8
Diet 2	75.0 (0.15)^{a,b}	113 (1.6)^{b}	124 (0.25)^{b}	53 (0.3)^{a}	347 (0.4)	57 (1.3)^{b}	19.4 (0.09)^{a}	17.9
Diet 3	77.8 (0.09)^{b}	126 (1.7)^{c}	113 (0.16)^{a}	48 (2.3)^{b}	322 (6.4)	41 (0.8)^{a}	19.0 (0.10)^{a,b}	17.0
Diet 4	82.9 (1.32)^{c}	134 (4.3)^{c}	92 (0.12)^{c}	33 (0.2)^{c}	218 (5.8)	55 (1.2)^{b}	18.7 (0.07)^{b}	11.7
G. edulis	95.4 (1.70)^{d}	ND	890 (1.02)^{d}	59 (2.3)^{d}	164 (1.6)	8 (1.4)^{c}	9.0 (0.24)^{c}	18.2

Water stability is expressed here as the percentage of dry weight remaining after overnight immersion in seawater. Protein leached is expressed as the weight of dissolved protein per kg of CP after 7 h immersion in seawater. Moisture is expressed as the weight (g) lost after 12 h at 105 °C. Ash, crude protein (CP), and total lipid (TL) values are expressed as g/kg of dry diet. Gross energy (GE) is expressed as MJ per kg of dry sample. Numbers in parentheses are standard deviations of the mean. Values in the same column that share the same superscript letter do not differ significantly ($P > 0.05$).

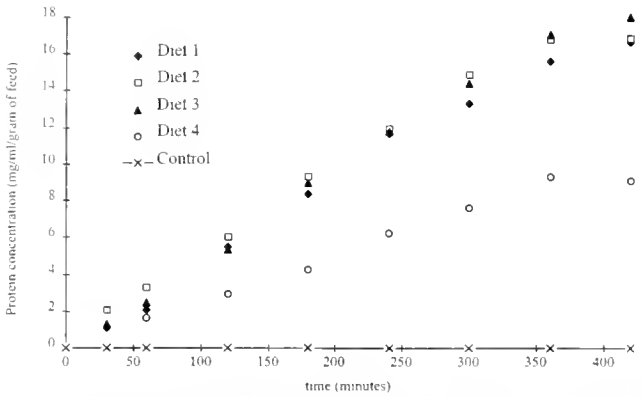

Figure 1. Rate of protein leaching measured in four formulated diets over 7 h. Values are expressed as milligrams of dissolved protein × mL of leachate^{-1} × gram of feed^{-1}. Error bars are omitted for clarity of presentation. The control consisted of three flasks of seawater treated in exactly the same way as the diet treatments.

of isoleucine, leucine, methionine, threonine, and valine and a lower amount of histidine (Table 2). Of the formulated diets, diet 4 contained the lowest amounts of all essential amino acids except for lysine (Table 2).

Fatty Acids

Large differences were found in the FA profile of *G. edulis as* compared with the four formulated diets (Table 3). The largest differences were between 20:4n–6 (53–79 times less abundant in the formulated diets) and 18:2n–6 (40–53 times more abundant in the formulated diets). Other large differences were seen for 18:3n–3, 20:1n-9 and 22:6n–3, which were less abundant and for 20:3n–6, which was more abundant in *G. edulis* as compared to the formulated diets. These differences culminated in total n–3 FAs being approximately five times higher in the formulated diets. Total n–6 FAs occurred at similar concentrations in all diets; whereas, saturated FAs (SFAs) were higher in *G. edulis* relative to the formulated diets. Differences in the FA profiles between formulated diets were less pronounced than those between *G. edulis* and formulated diets. The most obvious difference was with diet 4, which was higher in total n–3 and lower in total n-6 FAs compared to diets 1 to 3 (Table 3).

TABLE 2.

Concentrations of amino acid residues in *Haliotis asinina* tissue and four formulated diets and *Gracilaria edulis*.

Nonessential	*H. asinina*[a]	*G. edulis*[b]	Diet 1[b]	Diet 2[b]	Diet 3[b]	Diet 4[b]
Alanine	49.21	7.55	4.16	4.91	5.05	4.46
Aspartic acid	25.19	6.62	6.18	7.06	7.83	5.39
Glutamic acid	6.59	9.36	15.59	14.92	18.91	18.20
Glycine	96.63	7.65	5.18	6.02	5.71	5.54
Proline	47.44	4.98	7.62	6.93	7.23	7.54
Serine	37.34	6.33	5.49	5.53	5.79	4.83
Tyrosine	11.09	0.69	0.49	0.68	2.14	0.22
Essential						
Arginine	32.72	3.05	2.91	2.52	3.16	2.51
Histidine	5.99	0.83	1.67	1.77	1.65	1.27
Isoleucine	17.11	3.10	2.45	2.38	2.42	1.91
Leucine	30.55	5.29	4.56	4.73	4.80	4.27
Lysine	21.80	2.39	2.30	2.54	2.69	2.49
Methionine	12.00	1.11	0.81	1.01	0.81	0.53
Phenylalanine	9.22	1.59	1.61	1.46	1.44	1.35
Threonine	31.79	4.05	3.81	3.35	3.85	2.94
Valine	27.19	5.30	4.12	4.38	4.28	3.55

Values are the means of six abalone samples and two diet samples.
[a] Values expressed as g/kg.
[b] Values expressed as g/100 protein.

TABLE 3.

Fatty acid content of four formulated diets and *Gracilaria edulis*.

Fatty Acid	Diet 1	Diet 2	Diet 3	Diet 4	*G. edulis*
14:0	3.8	2.4	4.0	3.6	1.2
16:0	20.8	19.1	21.1	18.8	43.2
16:1n-7	3.0	2.2	3.5	3.3	1.4
18:0	3.8	3.5	3.8	3.2	1.8
18:1n-9	12.1	14.5	12.0	15.7	6.4
18:1n-7	2.1	2.0	2.2	2.3	3.8
18:2n-6	31.5	32.2	29.8	24.0	0.6
18:3n-3	3.8	3.4	3.7	2.5	0.4
18:4n-3	0.9	0.9	1.1	1.4	0.0
20:1n-9	3.4	3.8	3.8	5.1	0.2
20:3n-6	0.0	0.0	0.0	0.0	3.2
20:4n-6	0.6	0.4	0.6	0.5	31.7
20:4n-3	0.6	0.6	0.7	1.0	0.0
20:5n-3	3.8	3.4	4.2	5.0	1.9
22:1n-9	1.9	2.5	1.7	2.7	0.0
22:5n-3	0.8	0.9	0.9	1.2	0.5
22:6n-3	5.1	5.7	5.0	7.1	0.4
Summary					
Total *n*-3 PUFAs	3.9	3.5	3.8	2.7	0.4
Total *n*-6 PUFAs	31.7	32.6	30.1	24.4	4.2
Total *n*-3 HUFAs	11.2	11.5	11.9	15.7	2.8
Total *n*-5 HUFAs	0.7	0.4	0.7	0.6	32.8
Total *n*-3	15.1	15.0	15.7	18.4	3.2
Total *n*-6	32.4	33.0	30.8	25.0	37.0
SFA	29.6	26.2	30.1	26.7	47.6
MUFA	22.8	25.7	23.6	30.0	12.3
PUFA	35.5	36.1	33.9	27.0	4.6
HUFA	12.0	12.0	12.5	16.2	35.5

Values are presented as the percentage of total lipid and are the means of two samples.

Feeding Study

Animals reared on *G. edulis*, diet 1 and diet 2 produced significantly (P <0.05) higher TWBW growth rates than animals reared on diets 3 and 4 (Table 4). *G. edulis* produced significantly (P <0.05) higher SL growth than all other diets, followed by diet 1, which was significantly higher than all the other formulated diets (P <0.05). Diet 2 produced significantly (P <0.05) higher SL growth than diets 3 and 4, which did not differ from each other (Table 4). No difference was detected between male and female growth in terms of TWBW and SL growth (data not shown). Although the cumulative monthly measures of TWBW and SL show a fairly linear growth rate over 24 weeks across all diets (Fig. 2), the monthly growth rates (Fig. 3) revealed a considerable amount of intermonthly variation. Most noticeable was the eleva-

TABLE 4.

Increase in total wet body weight (g) and shell length (mm), mean condition index, mortality rates, food conversion ratio, and nightly dry food intake of abalone reared on four formulated diets and *Gracilaria edulis* for six months.

	TWBW Growth	SL Growth	CI	Mortality	FCR	DFI
Diet 1	3.92 (1.418)[a]	7.2 (1.40)[a]	87.1 (1.16)[a]	0.25	1.4 (0.87)[a]	0.67 (0.090)[a]
Diet 2	2.30 (0.664)[a]	5.3 (0.98)[b]	85.3 (1.40)[b]	0.38	1.8 (0.80)[a]	0.77 (0.073)[a,b]
Diet 3	1.80 (0.661)[b]	4.2 (1.00)[c]	85.0 (1.15)[b]	1.25	1.5 (0.21)[a]	0.66 (0.163)[a]
Diet 4	0.96 (0.493)[b]	3.2 (1.11)[c]	81.9 (1.90)[c]	1.25	1.6 (0.47)[a]	0.75 (0.099)[a,b]
G. edulis	3.98 (1.068)[a]	9.6 (1.24)[d]	85.0 (1.16)[b]	1.00	1.6 (0.34)[a]	0.85 (0.100)[b]

Total wet body weight (TWBW) growth data was square root transformed before any statistical analysis was performed, but values presented here are untransformed. Condition index (CI) measures are the weight of soft tissue (viscera and muscle) expressed as a proportion of the total wet weight (soft tissue plus shell weight). Mortality is expressed as the number of deaths recorded for each diet as a proportion of that recorded for *G. edulis*. Food conversion ratio (FCR) was calculated as the ratio of dry feed eaten to wet weight gained. Dry food intake (DFI) is the weight of dry food eaten each night, expressed as a percentage of wet body weight. Values in the same column that share the same superscript letter do not differ significantly. Numbers in parentheses are standard errors of the mean, except for FCR and DFI where they are standard deviations.

tion in growth over mo 2 to 4 relative to mo 0 to 2, followed by the subsequent decline over months 4 to 6.

Animals fed diet 1 had significantly higher ($P < 0.05$) condition indices than those reared on all other diets; whereas, animals fed diet 4 had significantly lower condition indices than those reared on all other diets (Table 4).

FCRs did not differ significantly between diet treatments (Table 4). However, the power of this ANOVA to detect a difference was very low (approximately 16%). FCRs for the formulated diets ranged from 1.4 to 1.8; whereas, that for *G. edulis* was 1.6. During the period of FCR measurement, abalone fed *G. edulis* ate significantly more ($P < 0.05$) than animals fed diets 1 and 3; whereas, those fed diets 2 and 4 did not differ from *G. edulis* in the weight of food eaten. Animals fed diets 1 to 4 did not differ significantly in the weight of food eaten (Table 4).

The results of the multivariate regression analysis identified two nutritional parameters that explained a significant proportion of the variation in growth observed: CP:GE ratio and the rate of protein leaching. Overall, the regression equation was highly significant ($P < 0.01$) with an R^2 value of 0.63 (Table 5). The CP:GE ratio carried the most weight in explaining growth with a beta coefficient of 0.55; whereas, the inverse relationship between protein leaching and growth had a beta value of −0.39. The normal probability plot of residuals revealed a very strong linear associa-

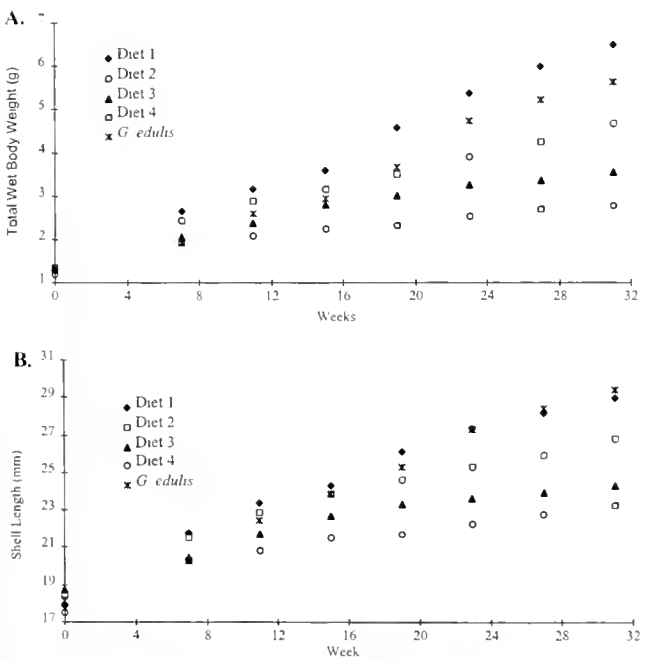

Figure 2. Average (n = 6) cumulative growth of abalone fed four formulated diets and *G. edulis* over 24 wk after a nutritional acclimatization period of 7 wk (shown as week 0 to week 7). Measures were taken every 4 wk. Growth was measured in terms of (A) total wet body weight and (B) shell length. Error bars are omitted for clarity of presentation.

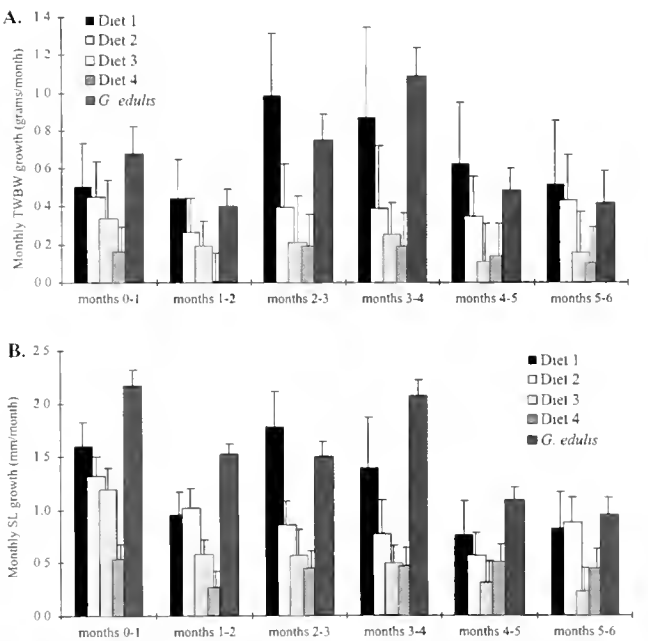

Figure 3. Average (n = 6) monthly growth rates of abalone fed four formulated diets and *G. edulis* over 24 wk after a nutritional acclimatization period of 7 wk. Monthly growth rates are presented in terms of (A) total wet body weight and (B) shell length. Error bars are the standard error of the mean.

TABLE 5.

Results of forward stepwise multivariate regression analysis.

Variable	Beta Coefficient	SE Beta	B coefficient	SE B	Tolerance	*P*-Value
CP:GE	0.55	0.13	0.09	0.02	0.83	0.0003
Protein leaching	−0.39	0.13	−0.04	0.01	0.83	0.006
Regression equation			Growth = 0.37 + 0.09 CP:GE − 0.04 Protein leaching			2.2×10^{-6}

tion, indicating that all variables were normally distributed. A scatter plot of observed versus predicted residuals revealed that a linear regression was a good description of the relationship between growth and the three selected variables. No outliers were detected.

Gonad Index and Histology

Males were significantly more mature than females by the end of the trial ($P = 0.029$). When separated by sex, the GI of females fed different diets did not differ significantly ($P = 0.23$); whereas, for males the effect of diet on GI was significant ($P = 0.015$); diet 1 produced the most mature males, followed in order by diets 2, 3, 4, and *G. edulis* (Table 6). The smallest animal with visible gonad tissue had an SL of 16.4 mm, weighed 0.92 g, was male, and had been fed diet 4 for 84 days.

When examined microscopically, all animals reared on *G. edulis* and diets 1 and 2 seemed to have healthy gonads when compared to gonadal sections of *H. asinina* taken by Apisawetakan et al. (1997) and Jebreen et al. (2000). Sections of gonads from animals reared on diets 3 and 4 showed evidence of substantial amoeboid cell activity and large areas of degenerated oocytes; animals reared on diet 4 seemed to be more affected than animals fed diet 3 (Fig. 4).

DISCUSSION

Growth Experiment

This study sought to compare the growth of *H. asinina* reared on commercially available Australian temperate abalone feeds relative to a *Gracilaria* diet, a genus of red algae known to sustain high growth in this haliotid (Upatham & Sawatpeera 1998, Bautista-Teruel & Millamena 1999, Capinpin et al. 1999). Comparing the growth rates achieved by *H. asinina* fed the commercial feeds to that of individuals fed *G. edulis* demonstrates that two of these formulated diets may be suitable for the aquaculture of *H. asinina* (Table 4 and Fig. 2). Although the highest SL growth was obtained on *G. edulis*, TWBW growth was not significantly different between *G. edulis*, and diets 1 and diet 2. From an aquaculture perspective, weight gain is considered to be more important than SL increase (see Fleming et al. 1996). Furthermore, the CI of animals reared on diet 1 (87.1%) was significantly higher than that of animals reared on *G. edulis* (85.0%), indicating that diet 1 produced healthy animals of a high product quality (Table 4).

TABLE 6.

Gonad index observations made at the end of the growth experiment.

	Diet 1	Diet 2	Diet 3	Diet 4	*G. edulis*	*P*-Value
Male GI	3	2	2	1.5	0	0.015
Female GI	0	1.5	1	1	0	0.225

Values are medians and range from 0 to 3, with 3 being fully mature. *P*-values were obtained from a Kruskal-Wallis analysis of variance (ANOVA) by ranks.

The diets and experimental conditions employed within this study produced growth rates lower than similar studies on *H. asinina*; Capinpin et al. (1999) recorded an SL growth rate of 60 mm/year (equivalent to 164 μm/day) with animals reared in sea cages and fed *Graciliaria bailinae*. Bautista-Tereul and Millamena (1999) achieved growth rates ranging from 222 to 247 μm/day over 90 days when 20 juvenile *H. asinina* (of a similar size to those used in the present study) were housed in large 60-L containers and fed either of three formulated diets. In the present study, *H. asinina* were reared in small (1.7 L) enclosures, which may have contributed significantly to the low growth rates (19–56 μm/day) when compared to these studies. Although the results are not directly comparable because of differing experimental conditions, Capinpin et al. (1999) found a significant density dependent effect on growth across four stocking densities: 43, 88, 130, and 175 abalone/m^2. The density used within this experiment equated to approximately 72 abalone/m^2, and implies that growth may have been affected by density. Furthermore, Mgaya and Mercer (1995) demonstrated a size grading effect on growth in *H. tuberculata* such that small abalone reared in the presence of large abalone did not grow as quickly as their size-graded counterparts. In our study, strict size grading did not occur. Growth may have been less variable and possibly higher had such a regimen been employed.

No significant differences were detected in the DFI by animals fed the formulated diets (Table 4), suggesting that differences in growth between these diets were attributable to nutritional factors rather than diet preferences (see Vandepeer *et al.* 1999). However, animals fed *G. edulis* ate significantly more than animals fed diets 1 and 3. Therefore, the possibility that animals fed *G. edulis* grew faster in terms of TWBW than animals fed diet 3 because of a higher food consumption cannot be ruled out.

Despite the short period over which FCR measurements were taken, the recorded values were low (1.4–1.8), and similar to those reported by Bautista-Tereul and Millamena (1999). The formulated diets used by these authors produced very high growth in juvenile *H. asinina* of a similar size to those used in the current study. Furthermore, Fleming et al. (1996) reviewed the food conversion efficiency (FCE) of seven diets formulated for temperate abalone by various feed manufacturers around the world and reported an FCR range of 0.77 to 3.33. The FCR values obtained in the present study compare favorably (Table 4).

All four formulated diets leached a considerable portion of their protein content when gently agitated at room temperature for 7 h (Fig. 1 and Table 1). The only other similar assay for abalone diets

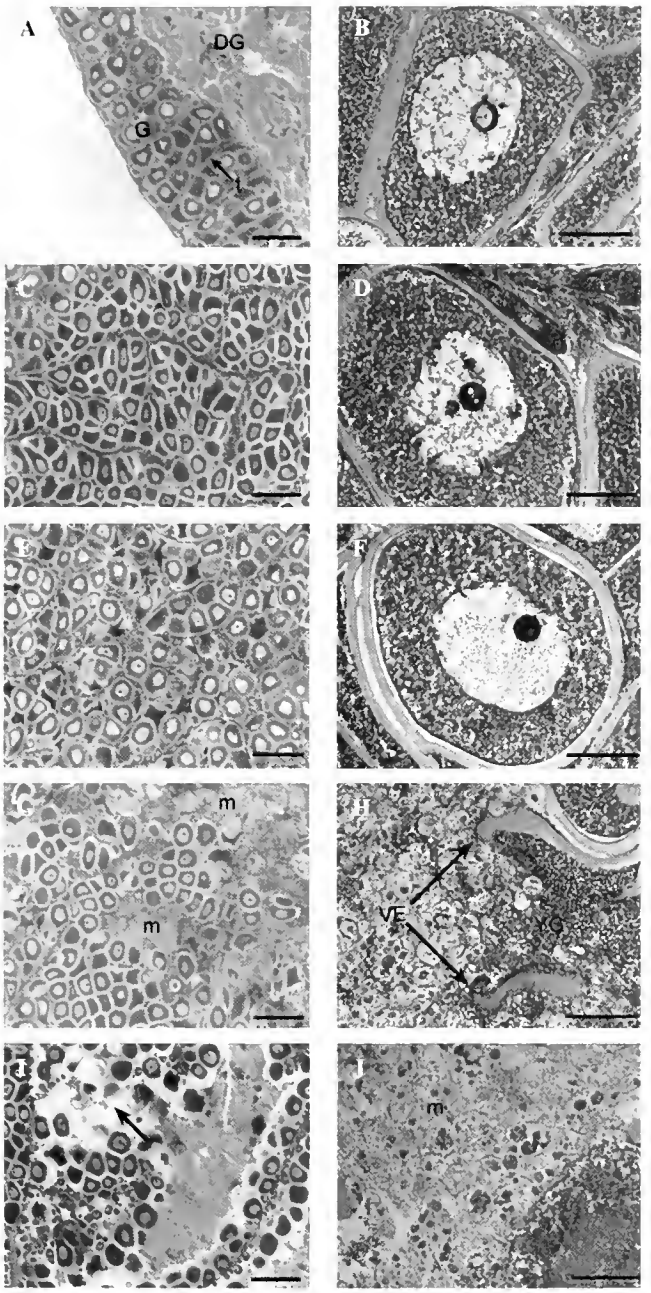

Figure 4. Representative ovary structures of *H. asinina* reared on a natural diet and four formulated diets. (A-B) Sections of ovary taken from an animal reared on *G. edulis* for 6 mo. Animals reared on *G. edulis* were not as mature as those reared on the formulated diets. This is reflected by the relatively thin layer of ovary that overlies the digestive gland. DG, digestive gland; t, trabeculae; G, Gonad. (C-D) Sections of ovary taken from an animal reared on diet 1 for 6 mo. Note the similarity in the over-all structure of the gonad in C, and in the size and shape of an individual oocyte and germinal vesicle in D. (E-F) Sections of ovary taken from an animal fed diet 2 for 6 mo. Note the similarities between diets 1 and G. edulis. (G-H) Sections of ovary taken from an animal fed diet 3 for 6 mo. Note the lack of over-all integrity of the gonad in G, with large populations of macrophages (m). In H, an oocyte that has had its vitelline envelope (VE) ruptured is undergoing phagocytosis by macrophages. Yolk granules (YG) are also evident. (I-J) Sections of ovary from an animal fed diet 4 for 6 mo. Note extensive voids left by degenerated gonad tissue (arrowhead) in I, and extensive macrophage activity in J. Scale bars in A, C, E, G, and I are 100 μm, and in B, D, F, H, and J are 10 μm.

(Viana et al. 1996) reported a leaching rate of 90.1 to 98.7mg/g over 12 h at 16°C, indicating that those diets were more protein-stable than those employed within the present study. The results of the protein leaching assay contradict the water stability results, because diet 4 (the most water-stable diet) leached the highest proportion of its protein; whereas, diet 1 (no different from the least water stable diet) leached the lowest proportion of its protein. This discrepancy between nutrient leaching and dry matter water stability has been noted by others (Viana et al. 1996, Edwards & Cook 1999).

The multiple regression analysis identified CP:GE and protein leaching rate as variables that explained a significant amount of the variation in growth between the five diets. The correct balance between energy and protein is essential for a diet to promote maximum growth (Fleming et al. 1996). Although CP:GE ratios are not as useful as digestible protein:digestible energy ratios (DP:DE), they were able to explain a significant amount of the variation in growth recorded in this study. The beta coefficients in Table 5 indicate the relative contribution of each variable to the regression equation. The standardized beta value of the CP:GE variable was higher than the protein leaching variable, indicating that CP:GE contributed the most toward the explanation of growth. The highest TWBW growth was achieved on diets *G. edulis* and diets 1 and 2, which had CP:GE ratios of 18.2, 20.8, and 17.9 MJ/kg, respectively. We expect that further research concerning the DP:DE ratio will allow the production of cheaper and more effective feeds (see Britz 1996, Britz & Hecht 1997, Fleming et al. 1996, Vandepeer et al. 1999). The different methods and ingredients used to bind a diet will presumably affect the rate that protein is lost from a diet. Because protein is essential for soft tissue growth in abalone and is an expensive component of a formulated feed, the efficiency with which a diet binds its protein could conceivably determine its over-all quality. The results of the multivariate regression analysis suggest that this is an essential characteristic of a formulated diet.

The two major differences in the FA content of *G. edulis* and the formulated diets lay in the amounts of 20:4n–6 and 18:2n–6 (Table 3). 20:4n–6 was 53 to 79 times more abundant in *G. edulis* than the formulated diets; whereas, 18:2n–6 was 40 to 54 times less abundant in *G. edulis*. Floreto et al. (1996) suggested that abalone may not be able to synthesize 20:4n–6 from the n–6 series of lower FAs and that it may be a nutrient that affects growth. This is supported by other workers (Dunstan et al. 1996, Mai et al. 1996, Uki et al. 1986). Although the percentage of 20:4n–6 in the formulated diets was much lower than in *G. edulis*, the higher measures of TL in diets 1 to 4 may have meant that this FA was adequately supplied.

18:2n–6 has been shown to enhance growth in other haliotids significantly (*H. discus hannai*, Floreto et al. 1996, Mai et al. 1996, and *H. tuberculata*, Mai et al. 1995). The obvious deficiencies of 18:2n–6 and total n–3 PUFAs in *G. edulis* may provide an explanation for the reduced growth rates noted in the present study in comparison to others studies on *H. asinina* that employed *G. bailinae* (see Bautista-Teruel & Millamena 1999, Capinpin et al. 1999). It could also partially explain the reduced growth rates noted during months 5 to 6 (Fig. 3A and B). It would be informative to analyze the FA profile of *G. bailinae* for comparison with *G.*

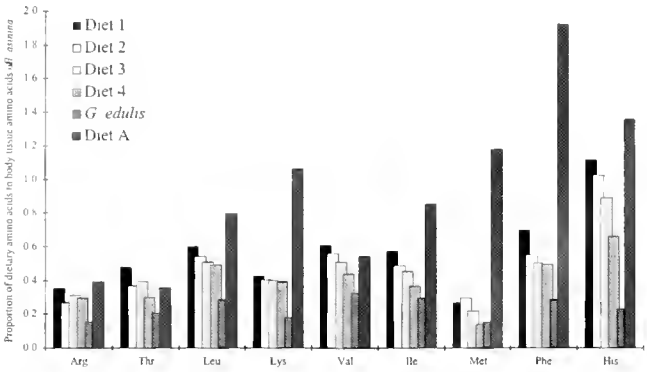

Figure 5. Essential amino acid profiles of 5 formulated diets and *G. edulis* (diet A was taken from Bautista-Teruel & Millamena 1999). The amount of each amino acid is expressed as a unitless proportion of the corresponding amino acid found in *H. asinina* soft body tissue.

edulis. It is also interesting to note that among the formulated diets, the amounts of 18:2n–6 in diets 1 and 2 (which promoted significantly higher TWBW growth) were higher than in diets 3 and 4.

Barneveld et al. (1998) concluded that Australian abalone feed formulators are oversupplying the lipid content of their feeds. Not only does lipid contribute to the energy content of a diet formulation (Fleming et al. 1996) affecting the DP:DE ratio, it may also inhibit the utilization of other nutrients (Barneveld et al. 1998). TL content was not detected by the multivariate regression analysis as being a nutritional parameter that affected growth. However, its putative inhibitory effect on nutrients (such as vitamins, minerals, and digestible energy) was not considered in this analysis. Diets 2 and 4 contained the highest amounts of TL. Furthermore, diet 4 contained the lowest CP content of the formulated diets. The low growth rate produced by diet 4 may be a result of the inhibitory effect of a high lipid inclusion on digestible protein. *G. edulis* contained a lipid concentration of 0.8% and produced high growth rates. This suggests that a diet with a blend of appropriate FAs may contain a minimal lipid content. The Japanese feed produced by the company Nihon Nosan Kogyo K.K. (NNKKK), which is reported to be a highly nutritious and well-balanced feed for abalone, contains a lipid content of 1.5% (Fleming et al. 1996), supporting evidence that abalone do not require a diet high in lipids.

Allen and Kilgore (1975) determined the essential amino acids required by *H. rufescens*. These 10 amino acids have since been assumed to be essential for other haliotid species (Mai et al. 1994, Knauer et al. 1995, King et al. 1996), and are assumed to be essential for *H. asinina* in the current study. The ideal ratio of amino acids in dietary protein has been shown to reflect the ratio of amino acids in the body tissue of fish (Wilson & Poe 1985). This approach to abalone diet formulation has been used in several studies (Knauer et al. 1995, reviewed in Fleming et al. 1996, King et al. 1996), although its accuracy when applied to abalone nutrition has not been rigorously tested. However, a comparison of the amino acid profile of the formulated diets and *G. edulis* to that of *H. asinina* soft body tissue seemed to explain some of the observed variation in growth between diets. The essential amino acid profiles of the five diets were compared by expressing each amino acid (g/kg) as a proportion of that found in the soft tissue of *H. asinina* (Fig. 5). Included in the comparison is the amino acid profile of a formulated diet (diet A) that was reported by Bautista-Tereul and Millamena (1999) to produce a very high growth (247 μm/day) in similar sized *H. asinina*. This diet was included to test the validity of this comparison. According to this method of comparison, a diet with consistent ratios of essential amino acids will provide adequate amounts of each residue; a diet with a flat profile, will be more balanced than a diet with any large fluctuations between residues. Such factors as the amount of protein in each diet, the feed intake, digestibility, and water stability will affect the over-all amount of each amino acid available to the abalone, and hence the height of each column. From Fig. 5, none of the diets within the current study had essential amino acid profiles that seemed to be very balanced. The essential amino acids methionine and arginine seemed to be limiting in diets 1 to 4 and *G. edulis*. A deficiency in arginine and methionine was reported by Mai et al. (1994) in six species of macroalgae, and also seems to apply to *G. edulis*. Lysine also seemed to be deficient in *G. edulis*. The relatively high concentrations of methionine, phenylalanine, and lysine in diet A as compared to diets 1 to 4 and *G. edulis* is obvious and may explain the high growth rates achieved by Bautista-Tereul

TABLE 7.

Essential amino acid profiles expressed relative to lysine for seven haliotid species.

Amino Acid	*H. asinina*	*H. discus*[a]	*H. iris*[b]	*H. midae*[c]	*H. tuberculata*[a]	*H. rubra*[d]	*H. rufescens*[e]
Arginine	1.50	1.35	1.69	1.27	1.22	1.70	1.21
Threonine	1.46	0.65	0.73	0.80	0.68	0.80	0.77
Leucine	1.40	1.14	1.24	1.11	1.15	1.16	1.31
Lysine	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Valine	1.25	0.76	0.69	0.74	0.78	0.80	0.79
Isoleucine	0.78	0.70	0.63	0.66	0.74	0.70	0.63
Methionine	0.55	0.31	0.26	0.34	0.32	0.38	0.43
Tyrosine	0.51	0.51	—	—	0.59	—	—
Phenylalanine	0.42	0.59	0.71	0.60	0.58	0.60	0.68
Histidine	0.27	0.29	0.36	0.29	0.29	0.34	0.33
Cystine	—	0.17	—	—	0.24	—	—
Met + cys	—	—	0.50	0.72	—	0.65	0.55
Phe + tyr	0.93	—	1.25	—	—	1.13	1.32
Tryptophan	—	—	0.19	0.13	—	0.18	0.06

[a] Mai et al. (1994). [b]Promak Technology (NZ) Ltd. as cited in Fleming et al. (1996). [c]Knauer et al. (1995). [d]King et al. (1996). [e]Allen and Kilgore (1975).

and Millamena (1999). However, the different experimental conditions between these two studies must be considered when comparing these growth rates.

When the essential amino acids of *H. asinina* are expressed relative to lysine, the profiles of all haliotids seem to be similar, except for higher amounts of threonine, valine, and methionine and lower amounts of phenylalanine *H. asinina* (Table 7). This supports the conclusions of previous comparisons between other species (reviewed by Fleming et al. 1996) and suggests that if diets formulated for temperate abalone can be supplemented with threonine, valine, and methionine, they will be suitable for *H. asinina*.

Gonad Index and Histology

The effect of diet on sexual maturation was not initially intended to be a measure of the nutritional quality of each of the diets investigated in this study. However, after some animals displayed gonad tissue early on in the trial, it was decided to make further observations. Although the effect of diet on gonad maturation, as assessed by a noninvasive method, was not statistically significant for females (Table 6), the size at which females on the formulated diets started to mature was smaller than females reared on *G. edulis*. The size at which animals started to mature was well below that reported by other workers (Singhagraiwan & Doi 1992, Capinpin et al. 1998); the smallest animal recorded with the first signs of gonad tissue was a male with an SL of 16.40 mm and weighing 0.917 g. Subsequent monitoring of individual abalone revealed monthly fluctuations in the presence and absence of gonad tissue (data not shown), indicating the ability of *H. asinina* to resorb germinal tissue. This was reflected in the results of the histological analysis. Female animals reared on diets 3 and 4 showed marked differences in the over-all gonad integrity when compared to those on diets 1, 2, and *G. edulis*. The lack of structure of gonad tissue among female animals on diets 3 and 4 (Figs. 4D and E) is reflected by the large populations of amoeboid cells evident in these gonads (Figs. 4I and J). In normal ovaries, amoeboid cells only proliferate after a spawning event and seem to be responsible for the degradation of unspawned mature oocytes (Jebreen et al. in press). Because of the random distribution of amoeboid cells and immature eggs within the ovaries of animals fed diets 3 and 4, it is assumed that this resorption of eggs is not a postspawning event, but is rather a response to either stress or malnutrition. Female animals reared on diets 1, 2, and *G. edulis* did not possess extensive populations of amoeboid cells in the gonads. There were no apparent histological differences in the gonads of male animals reared on different diets.

The precocious maturation by *H. asinina* maintained on formulated diets is an obvious concern for potential *H. asinina* farmers and feed formulators. Bautista-Teruel and Millamena (1999), achieved very high growth rates with *H. asinina* maintained on three formulated diet and noted no sexual maturation during the course of the 90-day experiment. Conceivably, a longer experiment would have seen reduced growth rates and yielded mature animals. This possibility is supported by Capinpin and Corre (1996), who found that all juveniles reared on a formulated feed and *G. heteroclada* were sexually mature at the end of a 120-day experiment. They attributed reduced growth rates toward the end of the experiment to a channeling of energy into gonad maturation. Fleming et al. (1996) suggested that the nutritional lipid requirement of abalone may increase during gonad development. Given the relatively high lipid content of the formulated diets tested within this study as compared to *G. edulis*, it is not surprising that female animals reared on the formulated diets were more mature than animals fed *G. edulis*. Ideally, feed formulations for "grow-out" diets should maximize somatic growth and minimize germinal growth. Further research in this area with more appropriate experimental designs (i.e., increased sample size and reduced handling stress) should yield a better understanding of the relationship between growth and reproduction in haliotids.

Conclusion

The results of this study indicate that *H. asinina* can be efficiently farmed on Australian diets formulated for temperate abalone. Efficient binding of nutrients into the diet to increase water stability, although retaining their biological availability for digestion still remains a challenge for feed formulators and seems to be an area of research that has not received the attention it deserves. The rates of protein loss from the formulated diets recorded in this study highlights the need for appropriate on-farm feeding strategies. Further research and development of these diets in terms of the DP:DE ratio, the amino acid requirements, and FA requirements will increase growth, making the culture of this species more profitable. On the basis of these results, *H. asinina* seems to be an ideal species for aquaculture within Australia; its highly frequent, synchronous and predictable natural spawning pattern (Counihan et al. 2001), very high meat to shell weight ratio (>85%), and high growth rate potential in culture (Bautista-Teruel & Millamena 1999, Capinpin et al. 1999) meet the major requirements for the economic production of any haliotid (Hahn 1989a).

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THE PARTIAL AND TOTAL REPLACEMENT OF FISHMEAL WITH SELECTED PLANT PROTEIN SOURCES IN DIETS FOR THE SOUTH AFRICAN ABALONE, *HALIOTIS MIDAE* L.

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ABSTRACT As protein is the most expensive nutrient to supply in abalone feeds, it is necessary to evaluate the relative performance of different protein sources in formulated diets. Fourteen diets were formulated to contain 34% crude protein and 6% lipid. These were fed to juvenile abalone, *Haliotis midae* (initial shell length: 10.6 ± 0.1 mm). Dietary fishmeal was substituted at 30, 50, 75, or 100% with plant protein concentrates, and the growth and nutritional parameters were recorded over a 180-day growth period. No significant differences were found in the growth rates between the control diet (100% fishmeal) and diets in which 30% of the fishmeal component had been replaced by either soy or sunflower meals, or torula yeast ($P > 0.05$). Substitution of 50% fishmeal with either soya meal or spirulina did not affect growth rates ($P > 0.05$). Replacement of either 75 or 100% of the fishmeal with plant protein sources had a significant affect on growth ($P < 0.05$). Pearson product moment correlations between dietary lysine levels and either growth rates or protein efficiency ratios revealed positive correlations ($r = 0.75$, $P < 0.002$; $r = 0.88$, $P < 0.0001$, respectively), suggesting that lysine may have been the first limiting amino acid in these diets. Carcass analysis revealed that dietary protein source had no significant affect on body composition ($P > 0.05$).

KEY WORDS: abalone nutrition, fishmeal replacement, plant proteins, *Haliotis midae*

INTRODUCTION

Increasing concern about the future supply and demand for fishmeal (Barlow 1989, Rumsey 1993) has increased efforts to reduce its use as the major protein source in commercial aquaculture feed formulations. The reduction of the fishmeal component in feeds has met with considerable success in a number of species, and suggests that its partial substitution with alternative protein sources need not compromise growth. The most notable success to date is illustrated by the channel catfish industry, where the substitution of fishmeal with soy, meat, and blood meals has reduced current fish meal inclusion rates to as low as 0–4% as compared with >30% in pre-1975 formulations (Stickney et al. 1996). Although the level of substitution has not been as dramatic in other sectors of the industry, it has been demonstrated that significant reductions in dietary fishmeal are possible. Among others, these sectors include the salmonids (Pongmaneerat & Watanabe 1992, Moyano et al. 1992), tilapias (Jackson et al. 1982, Lim 1989a), and shrimp (Lim 1989b).

With respect to abalone nutrition, a number of studies have compared fishmeal-based diets with other single protein formulations (Uki et al. 1985a, Uki & Wantanabe 1986, Uki & Watanabe 1992, Viana et al. 1993, Britz 1996), and although these studies give an indication of which proteins may have potential to become fishmeal substitutes, Fleming et al. (1996) reviewed the nutritional composition of twelve currently available commercial and experimental dietary formulations, and found that of the eight diets that contained fishmeal, all contained supplementary proteins. However, no indication of the effects that these alternative protein sources had on growth and nutritional indices is available. Nevertheless, considering the success of previous growth trials using single plant protein sources, and the fact that Haliotids are naturally herbivorous animals with a digestive and enzymatic physiology equipped for processing plant materials (Knauer 1994), the prospects for replacing dietary fishmeal with plant protein sources are promising. The aim of this study was therefore to determine the effects that the partial and total replacement of dietary fishmeal with locally available plant protein sources has on growth, nutritional indices, and body composition of juvenile *H. midae*. The protein sources chosen for investigation were: soy and sunflower meals, spirulina, torula yeast, and corn gluten meal.

MATERIALS AND METHODS

Experimental Animals and System

The experiment was conducted at the Port Alfred Laboratory using water drawn from the mouth of the Kowie River estuary on the Indian Ocean (33°45′S; 26°00′E). A 10,000 L semirecirculating system was employed. Water quality was maintained using biological filtration and the replacement of 25% of the system volume per day. A 12 light:12 dark photoperiod was used throughout the experimental period. Temperature and salinity were maintained at 20 ± 1°C and 35 ± 1 ppt, respectively. Animals were housed in 24 fiberglass experimental containers (30 × 50 × 26 cm, 39 L volume, four water exchanges per hour). Within experimental containers, three replicate groups of animals were housed in perforated 2-L plastic jars. Water flow through the jars was maintained by the use of airstones.

Juvenile hatchery-reared abalone (10.6 ± 0.1 mm shell length, 0.26 ± 0.01 g weight) were used in the experiment. Before experimentation, the animals were fed exclusively on a fishmeal-based artificial diet (34% crude protein, 4.5% lipid) and acclimated to the experimental system for a 1 mo period. Three replicate groups of 30 abalone were assigned to each dietary treatment, and placed in the experimental system according to a randomized block design. The duration of the experiment was 180 days. Whole body wet weight and shell length measurements were taken at monthly intervals throughout the experimental period. Weight was recorded to the nearest 0.01 g using an electronic balance and shell length to the nearest 0.01 mm using vernier calipers. Before these measurements were taken, the animals were starved for a 24-h period to ensure that their last meal had been processed and did not affect the animal's weight. A 10% (w/w) solution of magnesium sulfate was used to anesthetize the animals during the measurement process.

Animals were fed daily at 1700 h, and the feed consumed over the experimental period was recorded and corrected for leaching. Correction factors for total solids leached were determined for each diet by placing 4 g of feed in a control container containing

no abalone and placing it in the aquaria for a 12-h period. Total solids leached were calculated as dry weight loss over this period. Feed conversion ratios (grams dry feed consumed /grams wet weight gain) and protein efficiency ratios (grams wet weight gain/ grams protein consumed) were calculated for all treatments. Feed consumption and condition factors for all replicate treatments were calculated according to Britz (1996).

Dietary Formulations

Thirteen experimental diets were formulated to contain 34% crude protein, 4.5% lipid; a fourteenth diet containing 34% LT-fishmeal and 4.5% lipid was used as a control (Table 1). Formulations between experimental diets varied such that the fishmeal component of the control diet was substituted at either 30, 50, 75, or 100% with the selected plant protein sources. The diets were bound using agar according to the method described by Knauer (1994). In summary, 20 g agar was boiled in 1,200 mL water for 30 sec and allowed to cool to 45°C. The agar was then mixed with 80 g of dietary mixture, spread into a flat sheet 0.5-cm thick and allowed to set at room temperature. Once set, the diet was cut into squares (0.5 × 0.5 cm), oven dried at 60°C for 24 h and stored frozen at −20°C until required.

Carcass and Dietary Analysis

Proximate analyses of the dietary treatments and the abalone at the beginning and end of the experimental period were undertaken. Although triplicate 5-g samples of feed were used to determine the proximate composition of the dietary treatments; thirty animals at the start of the experiment and five animals from each replicate at the end of the experimental period were used to determine the proximate composition of the abalone. The abalone were initially frozen at −20°C for subsequent analysis. On defrosting, the animals were weighed and shucked, and the soft body material was homogenized using an Ika Turrax T25 homogenizer. With the exception of lipid, the proximate composition of both abalone and the diets were measured according to the standard methods of the AOAC (1984). Thus, crude protein was determined as total Kjeldahl nitrogen (N × 6.25), ash as the residue following the combustion of samples at 550°C for 12 h, and moisture by oven drying to constant weight at 104°C. Lipid was determined by the chloroform/ methanol extraction method (Folch et al. 1957), and the nitrogen-free extract was estimated from the difference.

Statistical Analysis

Growth rates were determined as the slopes of the natural log-transformed weight data. The slopes of the growth models were compared using analysis of covariance (ANCOVA) followed by a one-tailed F-test (Zar 1984). One-way analysis of variance and Student's–Newman-Keul's multiple comparison procedure were used to determine significant differences in feed consumption, condition factors, food conversion ratios (FCR), and protein efficiency ratios (PER) between dietary treatments, and, in addition, the effect of dietary treatment on the proximate composition of the abalone at the end of the experimental period. Comparisons between the essential amino acid profiles of the dietary treatments and the whole soft tissue of *H. midae* were undertaken using Pearson product moment correlations. In addition, Pearson product moment correlations were used to determine the strength of association between growth rates, protein efficiency ratios, and the levels of individual essential amino acids across all dietary treatments.

RESULTS

The partial and total replacement of dietary fishmeal with the alternative protein sources significantly affected abalone growth over the experimental period (Tables 2 and 3). Although five of the

TABLE 1.

Ingredient composition (% inclusion) and nutrient analysis of experimental diets; analysis expressed as a dry weight basis.

Diet	Control fishmeal 100 (%)	Fishmeal/ soy meal 70:30 (%)	Fishmeal/ soy meal 50:50 (%)	Fishmeal/ sunflower meal 70:30 (%)	Fishmeal/ sunflower meal 50:50 (%)	Fishmeal/ torula yeast 70:30 (%)	Fishmeal/ torula yeast 50:50 (%)
Danish LT fishmeal[a]	47.7	33.0	23.5	33.0	23.5	33.0	23.5
Soy meal[b]	—	22.5	37.5	—	—	—	—
Spirulina[c]	—	—	—	—	—	—	—
Sunflower meal[b]	—	—	—	28.6	47.7	—	—
Torula yeast[d]	—	—	—	—	—	23.8	39.7
Starch	31.3	21.5	15.4	15.8	5.9	22.2	15.0
Lipid[e]	—	2.0	2.6	1.6	1.9	0.9	0.8
Vit/min mix[g]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Agar[f]	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Analysis calculated							
Protein	34.2	34.2	34.2	34.2	34.2	34.2	34.2
Lipid	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Analysis (measured)							
Protein	35.1	35.6	33.0	31.5	33.3	31.4	32.2
Lipid	4.5	4.4	4.4	4.5	4.3	4.3	4.2
Ash	7.8	7.5	7.0	7.8	8.1	9.0	10.4
Moisture	5.2	6.4	6.5	6.3	5.3	6.8	6.2
Gross energy (MJ/Kg)	19.62	19.51	19.17	18.52	19.42	18.81	18.94
Leaching factor (solids)	0.96	0.81	0.81	0.91	0.93	0.91	0.92

continued on next page

formulations produced growth rates that were not significantly different to the control, the remaining eight formulations produced significantly lower growth rates. Growth rates and increases in abalone weight over the experimental period ranged between 1.09 to 1.41% body weight/day^{-1} and 600 to 1,230% respectively. FCRs and PERs ranged from 0.80 to 1.25 and 2.15 to 3.75 respectively.

With respect to the control diet (100% fishmeal), only torula yeast significantly improved feed conversion at both the 30 and 50% fishmeal substitution levels ($P < 0.05$, Table 2). However, although a 30% substitution of fishmeal with torula yeast did not significantly affect growth, at 50% substitution, growth was significantly lower than the control ($P = 0.00027$, Table 3). With respect to protein efficiency, a 30% substitution of fishmeal for torula yeast significantly improved efficiency ($P < 0.05$), but at a 50% substitution level, protein efficiency did not differ significantly from the control. In addition, consumption rates did not differ significantly between the control and the diets containing either 30 or 50% torula yeast.

In comparison with the control, the inclusion of sunflower meal at 30% or soy meal at either 30 or 50% did not significantly affect growth rates ($P > 0.016$, Table 3). Nevertheless, the inclusion of corn gluten meal at either 30 or 50% or sunflower meal at 50% produced significant reductions in growth rates ($P < 0.016$, Table 3). In comparison with the control diet, feed conversion was significantly improved when soy meal was used at the 30% substitution level ($P < 0.05$, Table 2) but did not differ significantly at a 50% level of substitution. In contrast, the opposite was found to be the case when either sunflower or corn gluten meals were used as substitutes. In both cases, a 30% fishmeal substitution produced an insignificant reduction in feed conversion ratio. At the 50% substitution level, feed conversion was further reduced, and in both cases, found to be significantly lower than control ($P < 0.05$). With respect to protein efficiency, inclusion of either soy, sunflower, or corn gluten meals produced a significant reduction in efficiency at both levels of inclusion. It is, however, interesting to note that at the 30% fishmeal substitution level, there were no significant differences in the protein efficiency ratios between the three protein sources. However, at the 50% substitution level, the protein efficiency ratios of the sunflower and corn gluten formulations were significantly lower than the soy meal formulation. At the 30% fishmeal substitution level, the consumption rates of these diets were higher than those observed for the control; however, it was only when inclusion rates were increased to 50% that they were significantly higher than the control ($P < 0.05$). One exception was the diet containing corn gluten meal, where a comparison with the control diet produced no significant difference in consumption rates ($P > 0.05$). As respective consumption rates and final body weights were generally found to increase and decrease across all the diets in which fishmeal had been substituted at either 30 or 50% with soy, sunflower, or corn gluten meals, a Pearson product moment correlation was performed between the consumption rates and final body weights to determine whether consumption rates were related to animal size. No significant correlation was found ($r = -0.42$, $P > 0.05$, $n = 7$); thus, it seems that differences in animal size cannot adequately account for the observed consumption rates. Differences in consumption rates may therefore be attributed to dietary factors.

The results using spirulina as a partial fishmeal replacement, demonstrated that a 50% substitution of fishmeal with spirulina produced neither a significant reduction in growth rate ($P > 0.0125$, Table 3), nor a significant reduction in feed conversion ratio (P

TABLE 1.

continued

Diet	Fishmeal/ corn gluten 70:30 (%)	Fishmeal/ corn gluten 50:50 (%)	Fishmeal/ soy meal/ sunflower meal 50:25:25 (%)	Fishmeal/ soy meal/ sunflower meal 25:37.5:37.5 (%)	Spirulina/ soy meal/ sunflower meal 50:25:25 (%)	Spirulina/ soy meal/ sunflower meal 25:37.5:37.5 (%)	Fishmeal/ spirulina 50:50 (%)
Danish LT fishmeal[a]	33.0	23.5	23.5	11.8	—	—	23.5
Soy[b]	—	—	18.8	28.1	18.8	28.1	—
Spirulina[c]	—	—	—	—	30.0	15.0	30.0
Sunflower[b]	—	—	23.9	35.8	23.9	35.8	—
Corn gluten[b]	15.9	26.6	—	—	—	—	—
Starch	28.3	26.7	10.5	0.5	3	0.8	23.9
Lipid[e]	1.8	2.2	2.3	2.8	3.3	3.3	1.6
Vit/min mix[g]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Agar[f]	20.0	20.0	20.0	20.0	20.0	16.0	20.0
Analysis (calculated)							
Protein	34.2	34.2	34.2	34.2	34.2	34.2	34.2
Lipid	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Analysis (measured)							
Protein	33.4	35.6	33.4	33.4	30.6	30.7	30.1
Lipid	4.7	4.8	4.2	4.2	4.2	4.1	4.2
Ash	5.9	5.0	7.8	7.6	11.2	9.5	11.4
Moisture	6.1	4.9	5.8	6.1	6.7	5.3	5.4
Energy (MJ/Kg)	20.29	19.36	19.65	19.23	19.65	19.23	18.27
Leaching factor (solids)	0.93	0.89	0.92	0.88	0.81	0.87	0.83

Dietary components supplied by: [a]Danish 999 fishmeals, Esbjerg Fiskeindustri, Denmark; [b]EPOL, S.A.; [c]Western Cape Tanneries, S.A.; [d]Illova Sugar, S.A.; [e]50/50 mixture of sunflower oil and "Marinol R" fish oil; [f]Continental Lab. Supplies, S.A.; [g]Composition from Uki et al. (1985b).

TABLE 2.

Growth and nutritional indices from juvenile abalone fed sixteen multiple protein diets; values are presented as means with standard errors.

Diet	Initial length (mm)	Final length (mm)	Initial weight (g)	Final weight (g)	Growth rate (%bd.wt.day^{-1})*	FCR	PER	Consumption (%bd.wt.day^{-1})	Condition factor		Mortality (%)
									Day 0	Day 180	
Control–fishmeal 100 (%)	10.9 ± 0.1	25.8 ± 0.3	0.26 ± 0.01	3.20 ± 0.12	1.38	0.92^{e}	3.39^{b}	0.89^{d}	1.14^{a}	0.99^{a}	0.0
Fishmeal/soy meal 70:30 (%)	10.8 ± 0.1	26.1 ± 0.3	0.27 ± 0.01	3.10 ± 0.09	1.31	0.82^{f}	3.02^{c}	1.11^{bcd}	1.20^{a}	0.99^{a}	1.1
Fishmeal/soy meal 50:50 (%)	10.6 ± 0.1	25.8 ± 0.4	0.26 ± 0.01	2.88 ± 0.10	1.32	0.88^{ef}	2.83^{cd}	1.27^{bc}	1.18^{a}	0.96^{abc}	1.1
Fishmeal/sunflower meal 70:30 (%)	10.5 ± 0.1	26.1 ± 0.3	0.25 ± 0.02	2.98 ± 0.09	1.37	1.00^{cde}	2.83^{cd}	1.23^{bc}	1.19^{a}	0.96^{abc}	2.2
Fishmeal/sunflower meal 50:50 (%)	10.6 ± 0.1	24.5 ± 0.3	0.26 ± 0.01	2.40 ± 0.09	1.23	1.20^{ab}	2.39^{def}	1.38^{abc}	1.20^{a}	0.91^{bcd}	0.0
Fishmeal/torula yeast 70:30 (%)	10.4 ± 0.1	26.7 ± 0.4	0.24 ± 0.01	3.48 ± 0.14	1.41	0.80^{f}	3.75^{a}	1.07^{cd}	1.19^{a}	0.94^{abc}	4.4
Fishmeal/torula yeast 50:50 (%)	10.9 ± 0.1	26.4 ± 0.4	0.27 ± 0.02	3.10 ± 0.12	1.27	0.86^{f}	3.12^{bc}	1.12^{bcd}	1.19^{a}	0.94^{abc}	0.0
Fishmeal/corn gluten 70:30 (%)	10.5 ± 0.1	23.1 ± 0.3	0.26 ± 0.01	2.39 ± 0.07	1.21	0.98^{cde}	2.84^{cd}	1.16^{bcd}	1.22^{a}	0.94^{abc}	3.3
Fishmeal/corn gluten 50:50 (%)	10.8 ± 0.1	23.1 ± 0.3	0.26 ± 0.01	1.58 ± 0.07	1.09	1.04^{cd}	2.36^{ef}	1.19^{bcd}	1.19^{a}	0.86^{d}	3.3
Fishmeal/soy meal/sunflower meal 50:25:25 (%)	10.7 ± 0.1	24.5 ± 0.3	0.27 ± 0.01	2.36 ± 0.08	1.19	1.06^{cd}	2.93^{c}	1.20^{bcd}	1.19^{a}	0.90^{bcd}	0.0
Fishmeal/soy meal/sunflower meal 25:37.5:37.5 (%)	10.8 ± 0.1	23.9 ± 0.3	0.26 ± 0.01	2.27 ± 0.09	1.17	1.03^{cd}	2.76^{cde}	1.31^{bc}	1.15^{a}	0.92^{abcd}	0.0
Spirulina/soy meal/sunflower meal 50:25:25 (%)	10.2 ± 0.1	24.4 ± 0.3	0.22 ± 0.02	2.27 ± 0.08	1.25	1.25^{a}	2.15^{f}	1.60^{a}	1.18^{a}	0.92^{abcd}	4.1
Spirulina/soy meal/sunflower meal 25:37.5:37.5 (%)	10.6 ± 0.1	24.4 ± 0.3	0.25 ± 0.01	2.32 ± 0.08	1.22	1.11^{bc}	2.43^{def}	1.42^{ab}	1.20^{a}	0.90^{cd}	4.4
Fishmeal/spirulina 50:50 (%)	10.5 ± 0.1	25.2 ± 0.3	0.26 ± 0.01	2.80 ± 0.09	1.28	0.97^{de}	2.89^{c}	1.23^{bc}	1.22^{a}	0.98^{ab}	3.6

* Whole body wet weight.

Different alphabetic superscripts within a column indicate significant differences between treatments for the parameter concerned. Growth rates are expressed as the slopes of the log-transformed weight data × 100. See Table 3 for ANCOVA describing significant differences between growth rates.

TABLE 3.

Analysis of covariance (ANCOVA) describing differences in growth rates between dietary treatments.

Code	Diet	Growth model	n	r^2	F-statistic	df	P	P > F
A	Control–fishmeal 100 (%)	ln y = −1.26 + 0.0138×	628	0.89				
B	Fishmeal/soy meal 70:30 (%)	ln y = −1.32 + 0.0131×	621	0.86	4.72^{AB}	1,1245	$P = 0.03005$	NS
C	Fishmeal/soy meal 50:50 (%)	ln y = −1.24 + 0.0132×	622	0.90	5.59^{AC}	1,1246	$P = 0.01823$	NS
					0.00^{BC}	1,1239	$P = 0.96$	NS
D	Fishmeal/sunflower meal 70:30 (%)	ln y = −1.38 + 0.0137×	602	0.90	0.16^{AD}	1,1226	$P = 0.68$	NS
E	Fishmeal/sunflower meal 50:50 (%)	ln y = −1.32 + 0.0122×	616	0.83	29.48^{AE}	1,1240	$P < 0.00001$	*
					27.63^{DE}	1,1209	$P < 0.00001$	*
F	Fishmeal/torula yeast 70:30 (%)	ln y = −1.28 + 0.0141×	613	0.82	0.55^{AF}	1,1237	$P = 0.46$	NS
G	Fishmeal/torula yeast 50:50 (%)	ln y = −1.19 + 0.0127×	614	0.85	13.31^{AG}	1,1238	$P = 0.00027$	*
					14.65^{FG}	1,1223	$P = 0.00013$	*
H	Fishmeal/corn gluten 70:30 (%)	ln y = −1.28 + 0.0121×	621	0.85	34.49^{AH}	1,1245	$P < 0.00001$	*
I	Fishmeal/corn gluten 50:50 (%)	ln y = −1.34 + 0.0109×	616	0.82	103.36^{AI}	1,1240	$P < 0.00001$	*
					17.22^{HI}	1,1233	$P = 0.00003$	*
J	Fishmeal/soy meal/sunflower meal 50:25:25 (%)	ln y = −1.23 + 0.0119×	621	0.82	39.04^{AJ}	1,1245	$P < 0.00001$	*
K	Fishmeal/soy meal/sunflower meal 25:37.5:37.5 (%)	ln y = −1.34 + 0.0117×	637	0.81	47.06^{AK}	1,1261	$P < 0.00001$	*
					0.43^{JK}	1,1254	$P = 0.51$	NS
L	Spirulina/soy meal/sunflower meal 50:25:25 (%)	ln y = −1.44 + 0.0125×	604	0.86	20.19^{AL}	1,1228	$P < 0.00001$	*
M	Spirulina/soy meal/sunflower meal 25:37.5:37.5 (%)	ln y = −1.33 + 0.0122×	600	0.87	31.38^{AM}	1,1224	$P < 0.00001$	*
N	Fishmeal/spirulina 50:50 (%)	ln y = −1.27 + 0.0128×	607	0.84	6.19^{AN}	1,1211	$P = 0.01298$	NS
					0.92^{LM}	1,1200	$P = 0.33$	NS
					2.76^{LN}	1,1187	$P = 0.096$	NS
					6.74^{MN}	1,1283	$P = 0.00956$	*

NS: Not significant.

*: Significant.

Growth models expressed as the natural log of the weight (y in grams) as a function of time (× in days) for the abalone fed the 16 experimental diets. Superscripts next to the F-values indicate which models were compared (n = number of values in model, r^2 = coefficient of determination, df = degrees of freedom, P = significance). To avoid accumulated error, significance was denoted at 0.0166 for three model comparisons, and 0.0125 for four model comparisons.

> 0.05, Table 2). Furthermore, although the animals fed this diet consumed significantly more feed than those animals fed the control diet ($P < 0.05$, Table 2); protein efficiency was significantly reduced ($P < 0.05$), indicating less efficient protein utilization. It is interesting to note that in the four diets containing the soy meal/sunflower meal combinations and fishmeal or spirulina at either 25 or 50% inclusion; feed conversion and protein efficiency were greater in those diets containing the fishmeal component as opposed to the spirulina. Nevertheless, a comparison between the diets containing spirulina or fishmeal at the 25% inclusion level, revealed that there were no significant differences between the feed conversion or protein efficiency ratios ($P > 0.05$, Table 2). However, at the 50% fishmeal or spirulina inclusion level, feed conversion, and protein efficiency were significantly reduced in those animals fed the spirulina-based diets ($P < 0.05$, Table 2). A comparison between the consumption rates revealed that at the 50% inclusion rate, those animals fed the spirulina-based diet consumed significantly more than those fed the fishmeal-based diet; however, at the 25% inclusion rate, consumption rates did not differ significantly.

Comparisons between the control and the diets containing the soy/sunflower combinations with fishmeal at either 25 or 50%, revealed that both formulations produced significant reductions in growth rates ($P < 0.00001$, Table 3), feed conversion, protein efficiency, and consumption ($P < 0.05$, Table 2). Nevertheless, it is interesting to note that between these formulations, no significant differences were found between either growth rates or the nutritional indices ($P > 0.05$, Tables 2 and 3).

To determine whether the dietary essential amino acid balance had an effect on growth and protein utilization by the abalone, a preliminary analysis using Pearson product moment correlations between the essential amino acid profile of the soft body tissues of *H. midae*, and the calculated essential amino acid profiles of the dietary treatments was undertaken (Table 4). The coefficients (r) from these correlations indicated that, with respect to the amino acid balance of the commercial formulation, and with the exception of those diets containing corn gluten, the amino acid balances of all the dietary treatments produced equal or closer approximations to the balance of amino acids found in the soft body tissues of the abalone. Secondary analyses, using Pearson product moment correlations were performed between the dietary/abalone EAA correlation coefficients and either the respective growth rates or the protein efficiency ratios. Because no significant correlations were observed ($P > 0.05$, Table 5), it can be assumed that, in this

TABLE 4.

Essential amino acid profile of the whole soft body tissues of *H. midae*, and the calculated essential amino acid profiles of the experimental diets.

	Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine + cystine	Threonine	Phenylalanine + tyrosine	Tryptophan	Valine	r
H. midae	**7.91**	**1.82**	**4.11**	**6.93**	**6.21**	**3.44**	**4.99**	**7.71**	**0.82**	**4.61**	—
Control–fishmeal 100	5.62	2.92	3.97	7.25	7.52	3.66	4.13	6.89	1.15	4.78	0.90
Fishmeal/soy meal 70:30	5.91	2.74	4.11	7.21	7.00	3.38	3.97	7.07	1.22	4.66	0.93
Fishmeal/soy meal 50:50	6.20	2.65	4.26	7.28	6.75	3.24	3.92	7.29	1.29	4.64	0.95
Fishmeal/sunflower meal 70:30	6.74	2.82	4.20	7.49	6.44	3.52	4.10	7.19	1.19	4.99	0.96
Fishmeal/sunflower meal 50:50	7.58	2.79	4.42	7.76	5.83	3.48	4.14	7.49	1.23	5.20	0.97
Fishmeal/torula yeast 70:30	5.49	2.83	4.48	7.16	7.48	3.37	4.46	7.72	1.11	5.11	0.91
Fishmeal/torula yeast 50:50	5.49	2.81	4.89	7.21	7.56	3.23	4.75	8.38	1.11	5.40	0.90
Fishmeal/corn gluten 70:30	4.87	2.70	4.32	10.07	5.76	3.73	3.85	7.43	0.94	4.84	0.80
Fishmeal/corn gluten 50:50	4.44	2.59	4.61	12.09	4.69	3.83	3.73	7.89	0.82	4.95	0.70
Fishmeal/spirulina 50:50	6.04	2.41	4.80	7.89	6.10	3.49	4.27	7.23	1.22	5.61	0.93
Fishmeal/soy meal/sunflower meal 50:25:25	6.95	2.74	4.37	7.58	6.33	3.59	4.06	7.45	1.27	4.96	0.97
Fishmeal/soy meal/sunflower meal 25:37.5:37.5	7.51	2.62	4.52	7.66	5.69	3.51	3.98	7.64	1.31	4.99	0.97
Spirulina/soy meal/sunflower meal 50:25.25	7.31	2.21	5.16	8.17	4.88	3.39	4.17	7.73	1.33	5.75	0.93
Spirulina/soy meal/sunflower meal 25:37.5.37.5	7.73	2.36	4.94	7.98	4.97	3.43	4.05	7.81	1.35	5.41	0.95

Comparison between dietary essential amino acid profiles and the essential amino acid profile of abalone are presented as the correlation coefficients between the dietary and abalone EAAs (r). Amino acid values are presented as a percentages of protein (dry weight).

The amino acid profiles of *H. midae*, spirulina, and torula yeast were taken from Britz (1995). The fishmeal amino acid profile was supplied by 999 Esbjerg Fiskeindustri, Denmark, and those of soy meal, sunflower meal, corn gluten, and maize taken from NRC 1993. Semolina was determined by the Department of Poultry Science, University of Natal, SA

study, dietary essential amino profiles could not be used to predict potential feed utilization by the abalone. In contrast, Pearson product moment correlations between the levels of individual dietary essential amino acids and either the growth rates or protein efficiency ratios (Table 5), revealed that dietary lysine levels were significantly and positively correlated to both growth and protein efficiency (growth rate: $r = 0.75$, $P < 0.002$; PER: $r = 0.88$, $P < 0.0001$). Furthermore, although leucine was found to be negatively correlated to the growth rate ($r = -0.68$, $P = 0.003$), and histidine positively correlated to protein efficiency ($r = 0.67$, $P < 0.05$), the other essential amino acids were not significantly correlated with either growth or protein efficiency ($P > 0.05$).

TABLE 5.

Pearson product moment correlations (r) between either the dietary essential amino acids or the correlation coefficients (r) between dietary/abalone EAA profiles, and growth rates and protein efficiency ratios.

	Factor			
Amino acid	**Growth rate**		**Protein efficiency ratio**	
Arginine	$r = -0.05$	$P > 0.05$	$r = -0.42$	$P > 0.05$
Histidine	$r = 0.41$	$P > 0.05$	$r = 0.67$	$P < 0.05$
Isoleucine	$r = -0.38$	$P > 0.05$	$r = -0.48$	$P > 0.05$
Leucine	$r = -0.69$	$P < 0.01$	$r = -0.46$	$P > 0.05$
Lysine	$r = 0.75$	$P < 0.002$	$r = 0.88$	$P < 0.0001$
Methionine +cystine	$r = -0.46$	$P > 0.05$	$r = -0.17$	$P > 0.05$
Threonine	$r = 0.47$	$P > 0.05$	$r = 0.46$	$P > 0.05$
Phenylalanine + tyrosine	$r = -0.43$	$P > 0.05$	$r = -0.22$	$P > 0.05$
Tryptophan	$r = 0.23$	$P > 0.05$	$r = -0.14$	$P > 0.05$
Valine	$r = -0.12$	$P > 0.05$	$r = -0.36$	$P > 0.05$
Correlation coefficients (r) between dietary and abalone EAA profiles	$r = 0.41$	$P > 0.05$	$r = 0.09$	$P > 0.05$

The body composition of the abalone at the end of the experimental period revealed that diet did not significantly affect the protein, lipid, carbohydrate (nitrogen-free extract), ash, or moisture levels in the soft body tissues of the animals ($P > 0.05$, Table 6).

Although condition factors were found to reduce in all treatments over the experimental period, at the end of the experiment, there were no significant differences in condition factors between the control diet and the experimental diets ($P > 0.05$, Table 2). The exceptions being those diets in which 50% of the fishmeal was substituted with either sunflower meal or corn gluten meal, and, in addition, those diets containing the mixture of soy and sunflower meals in combination with 50% fishmeal or 25% spirulina. In these diets, condition factors were found to be significantly reduced with respect to the control diet ($P < 0.05$).

Mortality of the abalone was low, ranging from 0–4.4%, and was attributed to the small animal size and the consequent susceptibility to damage caused by handling stress during weighing and measuring. There was no evidence of disease in any of the replicates.

DISCUSSION

Without exception, the substitution of dietary fishmeal with plant protein sources increased consumption rates in the abalone. However, because feed consumption may be affected by a number of factors, it is difficult to determine causality. Among others, factors that have been shown to affect consumption in poikilo-

TABLE 6.

Proximate composition (%) of the soft tissue of the abalone at the end of the experimental period.

Diet	Protein (%) ($n \times 6.25$)	Lipid (%)	Nitrogen-free extract (%)	Ash (%)	Moisture (%)
Control–fishmeal 100 (%)	55.4[a]	5.2[a]	26.1[a]	13.3[a]	74.6[a]
Fishmeal/soy meal 70:30 (%)	56.6[a]	5.4[a]	23.4[a]	14.6[a]	75.6[a]
Fishmeal/soy meal 50:50 (%)	54.9[a]	5.7[a]	26.5[a]	12.9[a]	76.6[a]
Fishmeal/sunflower meal 70:30 (%)	55.9[a]	5.1[a]	25.0[a]	14.0[a]	76.3[a]
Fishmeal/sunflower meal 50:50 (%)	52.8[a]	5.6[a]	28.1[a]	13.5[a]	75.2[a]
Fishmeal/torula yeast 70:30 (%)	55.3[a]	5.3[a]	25.1[a]	14.3[a]	75.6[a]
Fishmeal/torula yeast 50:50 (%)	55.9[a]	5.9[a]	23.6[a]	14.6[a]	76.3[a]
Fishmeal/corn gluten 70:30 (%)	54.6[a]	5.4[a]	25.8[a]	14.2[a]	77.9[a]
Fishmeal/corn gluten 50:50 (%)	51.6[a]	5.6[a]	29.1[a]	13.7[a]	75.6[a]
Fishmeal/soy meal/sunflower meal 50:25:25 (%)	54.6[a]	5.7[a]	25.8[a]	13.9[a]	74.9[a]
Fishmeal/soy meal/sunflower meal 25:37.5:37.5 (%)	56.9[a]	5.3[a]	23.5[a]	14.3[a]	75.8[a]
Spirulina/soy meal/sunflower meal 50:25:25 (%)	55.3[a]	5.5[a]	24.6[a]	14.6[a]	76.1[a]
Spirulina/soy meal/sunflower meal 25:37.5:37.5 (%)	51.0[a]	5.3[a]	29.9[a]	13.8[a]	77.2[a]
Fishmeal/spirulina 50:50 (%)	55.2[a]	5.4[a]	26.4[a]	13.0[a]	76.4[a]

With the exception of moisture, all values are expressed on a dry weight basis. Different alphabetic superscripts within a column indicate significant differences between treatments for the parameter concerned ($n = 5$, $P < 0.05$). Initial values for the abalone at the start of the experimental period were: protein—52.6%; lipid—4.1%; ash—15.2%; nitrogen-free extract—28.1; moisture—75.6% ($n = 30$).

therms include dietary energy density (Lee & Putman 1973, Weisberg & Lotrich 1982), and feed attractiveness and palatability (Harada et al. 1996).

If it is assumed that animals eat to satisfy their energy requirements (Smith 1989), it is reasonable to suggest that the differences in digestible energy across the dietary treatments provide an explanation for the observed consumption rates. It is possible that high levels of indigestible carbohydrates present in the plant protein sources may have reduced the dietary digestible energy densities. Thus, as the animals adjust their feed intake to satisfy their energetic requirements, they consume more of the fishmeal/plant protein diets that contain less available energy than the fishmeal-based diet. However, as it has also been demonstrated that dietary protein source may affect feed consumption in Haliotids (Uki & Watanabe 1986, Viana et al. 1994), the effect of differential attractiveness and palatability between protein sources cannot be discounted as factors that may have affected consumption.

The efficacy of soy meal as a partial fishmeal replacement has been well documented in fish (Reinitz 1980, Jackson et al. 1982, Watanabe et al. 1992), and although it has been incorporated into a number of formulations for Haliotids (Fleming et al. 1996), this is the first detailed information suggesting that it is a viable fishmeal replacement for use in diets for *H. midae*. Although protein efficiency was reduced at both the 25 and 50% substitution levels, growth rates were not significantly affected. Increasing dietary EAA deficiencies or lower bioavailability of nutrients may explain this reduction in protein efficiency. Although a number of studies have demonstrated that amino acid supplementation can improve nutritional indices in soy-based diets for salmonids (Dabrowska & Wojno 1977, Rumsey & Ketola 1975); methionine has been found to be deficient in soy-based formulations for Japanese eel (Lovell 1989) and carp (Murai et al. 1989). Therefore, it is probable that at the higher inclusion level, the soymeal-based diet may have been deficient in EAAs, and in particular methionine. However, it is not possible to verify whether this was the case until both the essential amino acid requirements of the species, and the availability of the amino acids in the soy and fishmeal have been established.

The growth and nutritional indices using the sunflower meal as a fishmeal replacement demonstrate that it has good potential for future use in formulations. Although feed conversion increased and protein efficiency reduced with respect to the control, the higher consumption rates may have improved growth rates, which, at the 30% fishmeal substitution level was not significantly different from the control. However, the reduction in growth and protein efficiency that became apparent when the inclusion level of the sunflower was increased from 30 to 50% may have been attributable to either a reduction in digestible protein or energy, or imbalances in essential amino acids. Although there is no information concerning the effects of sunflower meal in diets for Haliotids, there have been a number of studies with fish. Using rainbow trout, Sanz et al. (1994) cited imbalances between leucine and isoleucine as the primary factor reducing protein efficiency both in their study and an additional study carried out previously by Tacon et al. (1984). It is well documented that a leucine/isoleucine imbalance has a negative impact on growth and nutritional indices in fish (Harper et al. 1970, Hughes & Rumsey 1983, Robinson et al. 1984). However, until more detailed information regarding the essential amino acid requirements of abalone is available, it is not possible to determine whether this was the case in this study.

The combination of soy and sunflower meals as a partial fishmeal replacement significantly affected both feed conversion ratio and protein efficiency. These indices were respectively higher and lower than the control indicating poorer dietary utilization of the feed, which, with the exception of the 50% corn gluten substitution, produced the lowest growth rates in the study. Notwithstanding gross differences in digestible energy between the diets, these reductions in nutritional indices were probably caused by a combination of factors previously discussed when either the soy or sunflower meals were tested individually. It is, however, interesting to note that when the fishmeal component was completely removed and replaced with spirulina, there was an improvement in the growth rates. Not only does this suggest that spirulina is a viable fishmeal replacement—a result confirmed by the growth and nutritional indices obtained using the 50:50 fishmeal/spirulina

formulation—it also suggests that, as more information concerning the dietary requirements of the abalone and the availability of nutrients from plant protein sources becomes available, the complete removal of the fishmeal component may become a viable proposition.

The substitution of fishmeal with torula yeast produced some of the best growth rates and nutritional indices in the study. This was most apparent at the 30% fishmeal substitution level where the diet out-performed all the alternative formulations. The high apparent protein digestibility coefficient of the torula yeast (83.4% pers. obs.), combined with the high protein and feed efficiency suggests not only high availability of the protein source to the animals, but that it contains a well-balanced essential amino acid profile that supports good growth in this context. Nevertheless, at the 50% substitution level, the growth and nutritional indices were reduced with respect to the 30% substitution level. As consumption rates between the two formulations were not significantly different, this reduction in efficiency was probably caused by an imbalance of EAAs at the higher torula yeast inclusion level.

The results using the corn gluten meal suggest that it has potential as a fishmeal replacement at levels below 30%. Comparable studies with fish have indicated that inclusion levels between 20–30% dietary protein are viable, and do not have a negative impact on growth and feed efficiency (Gropp et al. 1979, Alexis et al. 1985, Robaina et al. 1997, Regost et al. 1999). However, in comparison with other protein sources, reduced feed efficiency and growth at higher inclusion levels (>30%) have been related to its poor essential amino acid profile, and, in particular, low lysine levels (Robaina et al. 1997). This may account for the results in this study in which both feed conversion and protein efficiency were reduced at the 50% inclusion level.

Although Pearson product moment correlations between the dietary and abalone EAA profiles revealed a large variation in correlation coefficients ($r = 0.70–0.97$, Table 4), Pearson product moment correlations between the r-values and the respective growth rates or protein efficiency ratios revealed no significant correlations (Table 5). Therefore, it appears that the balance of dietary EAAs was not a good predictor of either growth or protein utilization in the abalone. In contrast, when similar correlations were performed between growth, protein efficiency, and the levels of individual dietary EAAs (Table 5); the levels of dietary lysine were significantly and positively correlated to both growth rates and protein efficiency. Thus, although the bioavailability of the EAAs was not taken into account in this study, it can be concluded that, as opposed to the dietary EAA profile, dietary lysine levels provide a better indication of which protein sources will promote growth and protein efficiency. Furthermore, the positive correlations between dietary lysine levels and growth and protein efficiency suggest that lysine may have been the first limiting amino acid in these diets.

In conclusion, the results suggest that the potential to replace fishmeal with plant protein sources in commercial diets for *H. midae* is promising. However, although the commercial application of the torula yeast- and spirulina-based diets may be restricted by current high raw material costs from South African suppliers (respectively 340% and 250% higher than LT-fishmeal), the low raw material costs associated with the soy and sunflower meals make these protein sources particularly attractive to commercial feed formulators (respectively 60 and 75% lower than LT-fishmeal). Nevertheless, this study should be viewed as a preliminary investigation into the partial and total replacement of fishmeal; and that, before determining the optimal protein mixtures required to optimize growth at the least cost, a considerable amount of further research is required. In particular, the digestibility of essential amino acids and energy from the protein sources must be determined, the EAA requirements of the abalone, and the optimal dietary digestible protein: energy ratio. Only then can formulations be designed, which will optimize growth and feed utilization through the delivery of the required essential amino acids and appropriate digestible energy.

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THE USE OF STIMULANTS AS AN AID TO WEAN FISHERY CAUGHT BLACKFOOT ABALONE (*HALIOTIS IRIS*) TO ARTIFICIAL FOOD

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ABSTRACT Abalone use a combination of tactile and chemosensory feeding cues to detect suspended seaweed in their natural environment. However, in a commercial situation, adult abalone (*Haliotis iris*) caught for broodstock or pearling, show reluctance to start feeding on stationary artificial food. If they cannot be induced to feed, they may lapse into a starvation phase that can last several weeks. Subtidal adult *H. iris* (125 mm shell length) were collected during autumn, winter, spring, and summer from Banks Peninsula, Canterbury and held at Pendarves Abalone Farm Ltd. (South Island, New Zealand). Abalone were divided randomly into two groups. One group was offered a commercial pellet diet together with small quantities (0.03–0.05g dry weight L^{-1}) of suspended seaweed particles (*Gracilaria* spp.) to test its potential as a tactile phagostimulant. The other group (nonstimulant control) were supplied with the same commercial diet without the algal stimulant Each group consisted of three replicate vessels containing three individuals. Abalone behavioral responses and ingestion rates were monitored in both groups over a 3-wk period. Abalone in the stimulant experiment exhibited a typical feeding posture almost immediately, were more alert, and engaged in a greater range of feeding activities than the abalone that had been supplied with commercial food only. Those abalone without access to the algal stimulant remained either quiescent or alert in most seasons. Feeding activity of abalone in the stimulant treatment was significantly higher ($P < 0.05$) as compared with abalone from the nonstimulant control for all seasons except winter. Also, abalone consumed significantly more artificial food when tactile stimulants were present during autumn and winter. However, ingestion rates seemed highly sensitive to both season and acclimation time. Within each season, there was a consistent trend of increasing ingestion rate during the third week of the experiment, and this was most discernible in the stimulant treatment.

KEY WORDS: *Haliotis iris*, stimulant, artificial food, behavior, New Zealand abalone

INTRODUCTION

The commercially valuable New Zealand abalone, *Haliotis iris* Martyn, locally known as paua, is the main species of abalone harvested (Hooker & Creese 1995) and more recently, cultured in New Zealand (Clarke & Creese 1998). In addition to animals caught by the fishery for immediate processing, adult *H. iris* (≥125 mm shell length) may be taken to land-based holding facilities for later sale or, more critically, use in aquaculture as broodstock or for pearling.

Newly fished abalone are inevitably stressed by handling and air transport, and a holding system must, therefore, permit initial recovery and subsequently promote the growth and conditioning of gonads or allow abalone to become suitably physiologically robust to withstand the implanting of pearl nuclei. It is a major challenge to provide a suitable food source that will be accepted by the abalone throughout this period. Abalone are macroalgivores (Wee et al. 1992), using a combination of tactile and chemosensory stimuli to detect suspended and attached seaweed in their natural environment (Fleming 1995). Adult *H. iris* are largely sedentary, favoring exposed rocky substrata and feeding on drift seaweed detected by the cephalic and epipodal tentacles and subsequently trapped by the anterior foot or shell margin (Poore 1972a). Few algal harvesting permits have so far been granted for the commercial collection of seaweed in New Zealand (Schiel 1997), forcing farmers to rely upon artificial pellet feeds (certain artificial diets are also favored by pearl farmers for the resulting desirable nacre properties). Although nutritionally robust and relatively stable in seawater (Fleming 1995), the dense artificial pellets are unable to simulate the dynamic motion of neutrally buoyant seaweed fragments (Fleming et al. 1996). In the absence of the tactile stimulus of moving algae, captive *H. iris* will usually lapse into a starvation phase, taking up to 6 wk to commence feeding on pellets (pers. obs.); during this time, abalone inevitably suffer a loss of condition and may die.

Qualitative observations have suggested that the addition of small quantities of seaweed particles to a tank with suitably dynamic water movement will illicit a feeding response from adult *H. iris*, stimulating the abalone to graze upon static feed pellets that would otherwise be ignored on the tank bottom. This experiment is designed to characterize and quantify the nature of the abalone's response to the presence of a suspended tactile stimulant (dried *Gracilaria* spp. fragments). Seasonal variability in the feeding behavior of abalone is well documented (Poore 1972a,b, Shepherd 1973, Marsden & Williams 1996, Donovan & Carefoot 1998), hence the trials were repeated at 3-mo intervals over 1 yr and season treated as a fixed variable in subsequent analyses.

MATERIALS AND METHODS

Collection and Holding of Abalone

Adult *Haliotis iris* ≥125 mm shell length were collected using SCUBA from a depth of 6–10 m from a rocky reef within Whakamoa Bay, Akaroa Heads, New Zealand (43°53′S, 172°53′E). Collections were made once each season beginning in autumn (11 May 1999) and including winter (30 August), spring (4 November), and summer (22 December). The abalone were transported overland, in damp air at 5°C, to the Pendarves Abalone Farm Ltd., Canterbury, New Zealand.

Abalone were tagged and individual wet weight and shell length were recorded. Three animals were randomly assigned to each experimental vessel, an opaque 40-L tank receiving five exchanges h^{-1} of unfiltered seawater at ambient temperature (10–16°C), pH 8.2 ± 0.2, salinity 32 ppt.

Experimental Design

Two dietary treatments were examined. The first simply offered the abalone static pellets of artificial feed, while the second in-

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cluded *Gracilaria* spp. fragments suspended in the water column as a tactile stimulant. There were three replicate vessels for the stimulant treatment and the control group. Behavioral responses of abalone were quantified in both groups for an initial 10-h period, and ingestion rates were measured at 3-day intervals for 3 wk.

Tanks were covered with black polyethylene sheeting to provide total darkness for the initial 96 h after stocking, providing the abalone with an undisturbed 4-day recovery period from handling and transportation. On day 5, the covers were removed and the tanks siphoned clean. To three tanks were added 3 g of preweighed, dry artificial food (Abfeed™, Sea Plant Products, South Africa), these are referred to as the nonstimulant treatment or control. A further three tanks were supplied with 3 g Abfeed, plus 1.5 g of dried, mulched *Gracilaria* spp. particles (stimulant treatment) equivalent to 0.03–0.05 g L^{-1}. The tank inflow jet was positioned to suspend the *Gracilaria* spp. particles in the water column and leave the Abfeed pellets static on the tank floor.

Behavioral Responses

Abalone behavior was observed and recorded over a 10-h period beginning at approximately 1 PM and finishing at 11 PM. A photoperiod of 12 h light/12 h dark was maintained with lights being turned off at 6 PM. Abalone were observed at night under red light. Abalone behavior was divided into the following categories:

1. quiescent (shell clamped, cephalic, and epipodal tentacles retracted);
2. alert (shell slightly raised, epipodal, and cephalic tentacles extended);
3. locomotion (crawling);
4a. feeding on artificial pellets;
4b. feeding on *Gracilaria* spp. particles; and
5. active feeding posture (shell and foot raised, anterior lobes of foot and tentacles extended and waving).

Abalone were examined every 15 min and their behavior recorded. At the end of the 10 h period, nylon lids were placed on the tanks to minimize light-shadow disturbance. For the purpose of statistical analysis, behavioral responses were combined into feeding (categories 4a, 4b, and 5) or nonfeeding behavior (1–3).

Ingestion Rates

Tanks were cleaned and food and stimulant replaced every 3 days following the completion of the behavior assessment. Cleaning consisted of the inflow being turned off; all solid material was then siphoned from the bottom of the tank and collected on GF/C filter paper. The solids were rinsed in fresh water, and all identifiable food particles were isolated from the *Gracilaria* spp. and feces were then dried at 55°C for 24 h and weighed. Artificial feed was also stocked to a control tank containing no animals, the proportion of initial weight lost was used as a correction factor to compensate for passive leaching; in this way a correction factor was determined for each season using weight change over a 3-day period. Biomass-specific ingestion rates were calculated for each group of abalone during each 3-day feeding period using the following equation:

$$I = \frac{[(W_I \times C_{summer}) - W_F] \div 3}{B} \cdot 100$$

Where ingestion rate, I (percentage biomass ingested per day), is determined from W_I and W_F, the initial and final dry weight of artificial food over a 3-day feeding period corrected for C, the season-specific leaching correction, and standardized by biomass of abalone in the tank B.

Abalone ingestion rates were calculated over 3 wk for each seasonal sample, but this was extended to 4 wk in winter because of low rates of food intake.

Statistical tests were performed using Statistica™ 5.1 (StatSoft Inc., USA).

RESULTS

Behavioral Responses

During all four seasons, the abalone tended to be largely inactive (categories 1 and 2 on Fig. 1), spending less than 10% of the time moving about the tank (category 3). The presence of the algal stimulant had little effect upon the amount of time engaged in each

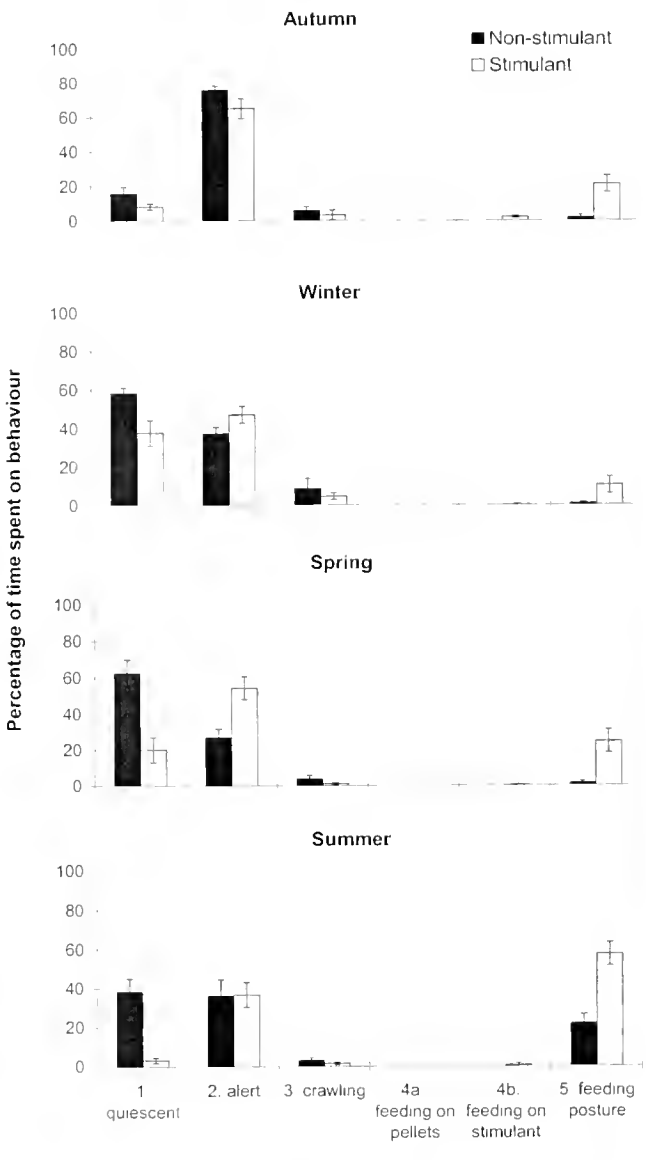

Figure 1. Seasonal time budgets for *Haliotis iris*, behavioral categories recorded over a 10-h period in the presence (Stimulant) or absence of algal stimulant (Nonstimulant). n = 9 individuals per treatment. Graphs show mean ± SE.

nonfeeding activity during autumn and winter (Fig. 1). However, abalone receiving the stimulant treatment spent relatively little time clamped and unreceptive during spring and summer (20.1 ± 7.0% and 3.3 ± 1.3%, mean ± SE, respectively) as compared to their nonstimulant counterparts (62.6 ± 7.2% and 38.5 ± 6.6%).

A two-way analysis of variance (ANOVA) was used to examine the effects of stimulant presence and season upon the amount of time devoted to feeding activities (categories 4a, 4b and 5). Both the stimulant ($F = 54.24$, $P < 0.001$) and season ($F = 21.99$, $P < 0.001$) had a significant effect on the duration of the feeding response, with no interaction between the two factors ($F = 1.13$, $P = 0.342$). Tukey's "Honest Significant Difference" pairwise comparison found the feeding activity of animals in the stimulant treatment was significantly higher ($P < 0.05$) than nonstimulant for all seasons except winter. Within each treatment, summer feeding activity was significantly higher than all other seasons. Nonstimulant abalone that exhibited virtually no feeding behavior between autumn and spring spent 22.0 ± 5.0% (mean ± SE) of their time in a feeding posture in summer. In contrast, abalone from the stimulant treatment spent 57.0 ± 6.0% of their time in this posture, as compared to 17.0 ± 3.5% throughout the rest of the year. With the exception of isolated instances, where stimulant particles were successfully captured by the abalone, all feeding activity during the initial 10-h observation period was limited to a receptive posture rather than actual ingestion.

Ingestion Responses

Abalone consumed significantly more artificial food when tactile algal stimulants were present during autumn and winter (ANCOVA, $F = 4.763$, $P = 0.035$, and $F = 7.650$, $P = 0.008$, respectively). It must be noted, however, that winter trials were extended for a fourth week because of very low ingestion rates. No significant differences were detected between stimulant and nonstimulant treatments in spring ($F = 2.397$, $P = 0.130$) and summer ($F = 2.159$, $P = 0.150$). Ingestion rates seemed highly sensitive to both season and acclimation time (Fig. 2). Despite erratic abalone consumption rates during autumn and very low initial rates in winter and spring, a consistent trend of increasing ingestion rate occurred during the final week of all of the seasonal experiments. This was most discernible in the stimulant treatment. A similar potential trend in summer may have been obscured because of a pump failure during the final 3 days of monitoring.

Because the ingestion response attributable to the presence of stimulants seemed to become more pronounced over time, the final 3-day ingestion data from each season were considered to be the most representative predictor of long-term feeding responses. Statistical analysis (two-way ANOVA) found that both treatment ($F = 3.4$, $P < 0.001$) and season ($F = 14.6$, $P = 0.002$) had a significant effect upon final ingestion rates; there was no significant interaction. A least-significant difference post hoc comparison of means (95% CI) found final ingestion rates were significantly higher in the presence of stimulants in autumn (0.13 ± 0.01% biomass d^{-1} in stimulant, 0.07 ± 0.007% biomass d^{-1} in nonstimulant), but no significant difference for any other season. An examination of seasonal effects alone, based upon final ingestion rate, ranked autumn significantly higher than summer, which was, in turn, significantly higher than winter and spring, which were similar.

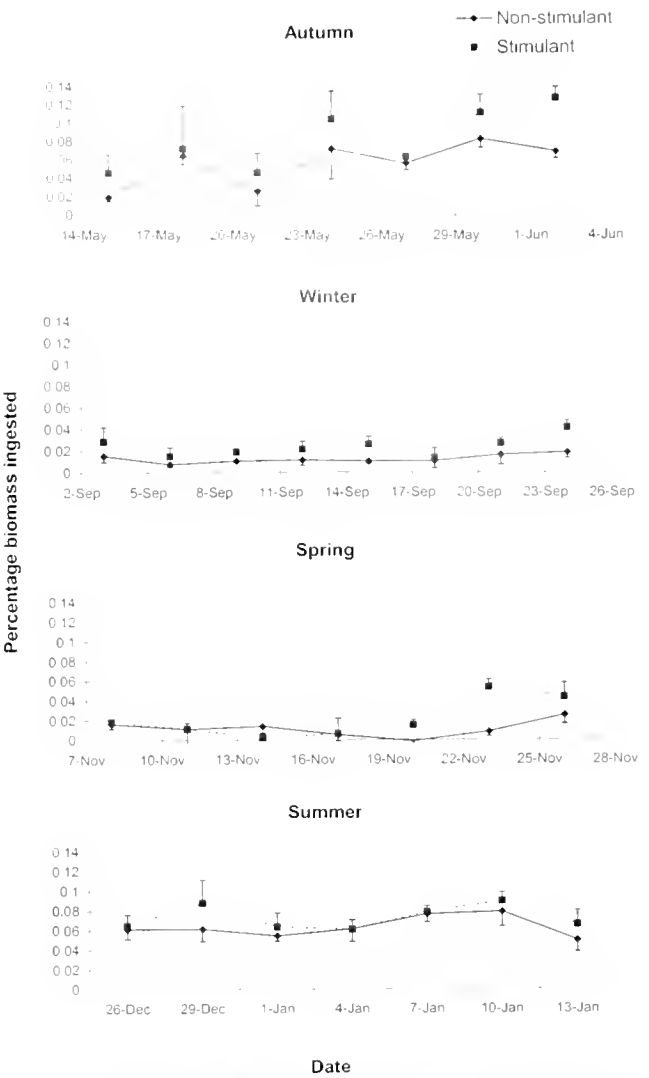

Figure 2. Seasonal ingestion rate of *Haliotis iris* provided with an artificial diet in the presence (Stimulant) or absence of algal stimulant (Nonstimulant). Graphs show mean + or −SE.

DISCUSSION

To understand the responses of wild abalone following capture and transfer to an artificial situation, such as a commercial facility, it is important to understand their feeding mechanisms and behavior. Initially thought to be browsing herbivores, haliotids tend to be largely sedentary except when food is scarce (Poore 1972a,b). Adult *H. iris* consume a wide range of seaweeds (Tunbridge 1967), feeding predominantly on drift algae in most localities (Schiel 1992). *Haliotis ruber* Leach and *H. laevigata* Donovan, when exposed to sufficient water flow, raise the front of their shells to catch drift seaweed with their foot instead of grazing on benthic algae (Shepherd 1973). It was noted that this posture also occurred in the absence of drift algae. Hingham et al. (1998) also described the behavior whereby abalone were observed to form two "hands" with their foot and grasp food as it moved past, detected by contact with the epipodal tentacles. Poore (1972a) noted that *H. iris* responded to drift seaweed in the same way.

Abalone use both chemosensory and tactile cues to detect food in the wild. Crofts (1929) observed that the cephalic, mantle, and epipodal tentacles appreciate even slight chemical changes in the water and that in all the tentacles, the sense of touch is particularly acute. The cephalic and epipodal tentacles play an important role in investigating the environment and guiding the abalone around their habitat (Crofts 1929, Upatham et al. 1998) and also respond to such tactile stimuli as drifting algal fronds and particles. The sensitivity to such stimuli is attributable to the cluster of sensory cells that reside beneath the epithelium on the tentacles, lips, anterior edge of the foot, and mantle edge (Luchtel et al. 1997). According to de Vlieger (1968), the optimal stimulus for positive thigmotaxis (during creeping as well as when exploratory movements are carried out) is a nonmoving object occasionally met by the foot (e.g., food pellet). This results in the withdrawal of the tentacle or foot followed by a full tentative exploration of the initial stimulus (de Vlieger 1968). Abalone in both the control and algal stimulant treatments were observed to touch and feel around the edge and over the whole pellet with their foot after they had physically encountered it (they usually bumped into it, then stopped to explore). Some abalone crawled over the pellets after exploring them, but no attempt at ingestion was made. Hence, contact with the food pellets seemed to elicit a positive thigmotactic response but did not stimulate a feeding response in freshly caught *H. iris*.

Abalone were regularly observed in a typical feeding posture in the tanks where stimulants were provided; however, this same feeding posture was also observed in the nonstimulant tanks, especially in summer. These abalone were found directly under the water in-flow with their shells raised and feet extended, corroborating the view taken by Shepherd (1973), who suggested that water movement is an important environmental factor affecting the feeding of those species that feed on algal drift. The increased tendency to adopt a feeding posture accounted almost entirely for the significantly elevated level of feeding behavior of *H. iris* in the stimulant treatment. Abalone consistently spent less time in the unreceptive quiescent/clamped state when stimulants were present (Fig. 1). Because clamping is usually regarded as an escape response in haliotids, this behavior may be considered a reaction to an unfavorable environment. In the presence of stimulants, the amount of time spent quiescent seemed closely related to water temperature. The same observation was found for *H. kamtschatkana* Jonas (Donovan & Carefoot 1998) and may reflect metabolic limitation rather than an avoidance response. It is interesting to note that captive adult *H. iris* do not share the wild population's tendency to increase foraging (crawling) rate in response to hunger (Poore 1972a, Donovan & Carefoot 1998), nor to the presence of stimulants, locomotion tending to be limited to negative phototactic response only (pers. obs).

The ingestion rates of *H.iris* feeding on a commercial pellet diet support the initial hypothesis that, with the addition of a tactile algal stimulant, abalone would be encouraged to feed on the artificial food and increase the ingestion rate. In all the trials; however, ingestion rates varied over time. Feeding rates were low or erratic for the first 2 wk of captivity, then increased steadily toward the end of each trial (Fig. 2.). This pattern occurred sooner and was better defined in the stimulant treatment than in the nonstimulant control. Ingestion rates increased up to 50% in autumn and winter and slightly less in spring and summer. The improvement in dietary intake of abalone in the presence of the stimulant usually began after 9–12 days and was consistently later in nonstimulant abalone, typically taking 15–18 days (Fig. 2.). Extended trials using a larger sample of individuals to establish the steady-state ingestion rates of abalone when fully acclimated to captivity and artificial food would be valuable in determining the long-term role of seaweed particles as a phagostimulant.

Differences in abalone ingestion rate between the four seasons can be attributed in part to elevated water temperature, which can increase metabolic rate and subsequently feeding rate (Marsden & Williams 1996). Hence, although it might be predicted that feeding rates should be higher during summer than in the autumn, food consumption may be affected by other metabolic demands, including the abalone reproductive cycle. *Haliotis iris* from the collection site are known to spawn during the autumn (March to May) (Poore 1972b); hence, increased food intake may be neccessary to support rapid gonad development (gonad condition was not examined to minimize handling stress). Some apparent inconsistency in the ingestion data may also have been caused by the collection times within each season. Autumn, winter, and spring collections were made relatively late because of the uncompromising dive conditions, and an early collection was made in summer to exploit favorable sea conditions.

Applied nutritional research on abalone has focused upon the use of attractants and chemical feeding stimulants. Harada et al. (1996) tested various novel ingredients, including herbs and spices for their attracting ability and Sakata and Ina (1992) used different algal methanol extracts to study the feeding behavior of young *H. discus*. Also, attempts have been made to design a neutrally buoyant feed that simulates algae in the culture tank (Fleming et al.1996), and more recently, the role of water movement in stimulating abalone feeding has become recognized (Hingham 1998). The results from the present study suggest that tactile algal particles could provide an additional stimulant, complementing those already described.

In conclusion, the addition of *Gracilaria* spp. particles to the water of a tank containing newly collected field specimens of *Haliotis iris* immediately stimulates a positive feeding response. This subsequently shortens the period to first acceptance of artificial feed and seems to stimulate elevated ingestion rates. The nature of these responses; however, is highly seasonal and may be subject to a period of acclimation during which the feeding response can change. It is suggested that this aspect be examined further using a larger sample size and individuals of a range of shell lengths. Also, investigations into the effects of tactile phagostimulants on ingestion and subsequent meat, gonad, and nacre development may assist the future development of the abalone culture industry.

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COMPARATIVE PERFORMANCES OF JUVENILE *HALIOTIS ROEI* FED ON ENRICHED *ULVA RIGIDA* AND VARIOUS ARTIFICIAL DIETS

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ABSTRACT Growth rates of juvenile *Haliotis roei* fed inorganically enriched *Ulva rigida* were compared with growth achieved on various artificial diets. Juvenile abalone (20–40 mm SL) were collected from reef platforms off the Perth (Western Australia) metropolitan area and assigned to one of seven different dietary treatments. All diets were fed *ad libitum* (3% bw day $^{-1}$) every second day, and growth rates were quantified over a 3-mo period. Specific growth rate (SGR) for both shell length and whole body weight indicated that growth of abalone fed enriched *U. rigida* was not significantly different ($P > 0.05$) to growth achieved from the best performing artificial diets.

A 96-h salinity stress test was also conducted on all treatments to assess the effects of diet on stress resistance. Diet significantly effected survival at 20‰, with the *U. rigida* fed animals exhibiting decreased tolerance to hypo-osmotic conditions ($P < 0.05$). This may have been caused by an interference with the cell volume regulatory mechanisms normally utilized by *Haliotis* spp.

Enrichment of wild *U. rigida* increased the algal protein content from 11.4 ± 2% (dry weight) to 32.2 ± 1.5%, perhaps partially explaining the difference in performance between this and other abalone feed trials utilizing *Ulva* spp. Results indicate that enriched *U. rigida* is a suitable feed for *H. roei*, providing similar growth to that achieved from several commercially available diets.

KEY WORDS: abalone, *Haliotis roei*, *Ulva rigida*, nutrition, salinity tolerance

INTRODUCTION

The main constituents of aquaculture effluent are ammonia and phosphate, with ammonia known to be the main contributor to marine eutrophication when added to an oligotrophic marine environment (Dosdat et al. 1995, Wu, 1995, Lemarie et al. 1999). Many shore-based aquaculture facilities utilize sedimentation ponds to remove particulate matter but ignore dissolved inorganics such as ammonia (NH_3/NH_4^+) and phosphate (PO_4^{3-}) because of the difficulty of their removal from high volumes of water. Use of macroalgal biofilters has been shown to remove a large proportion of the dissolved nitrogen from aquaculture effluent when integrated into intensive fish culture systems (Neori 1996, Shpigel et al. 1996a). Species of sea lettuce (*Ulva* spp.) have been shown to remove up to 90% of dissolved nitrogen from aquaculture effluent (Neori et al. 1998). Research in Israel has also shown that culture of *Ulva* spp. in nutrient-rich waters increases their protein content from 11–13% to over 35% of the dry weight (Shpigel et al. 1999). This enriched *Ulva* has been shown to provide good growth rates for *Haliotis tuberculata* (Neori et al. 1998, Shpigel et al. 1999), and *Haliotis discus hannai* (Corazani & Illanes 1998, Sbpigel et al. 1999). Aquaculturists may be enticed to adopt effluent treatment procedures more readily if shown that enriched *U. rigida* can also be used as a feed for abalone species.

Stress tests have previously been used as an assessment of general health or vigor in several aquatic species (Dhert et al. 1990a; Dhert et al. 1990b, Briggs 1992, Boarder & Maguire 1998, Samocha et al. 1998). Abalone are osmoconformers and possess only limited osmoregulatory capabilities; thus, the effects of salinity fluctuations directly affect internal ionic composition (Brix 1983, Somero & Bowlus 1983). It has been shown previously that dietary history can directly effect resistance to low salinity in abalone (Boarder & Maguire 1998).

This trial was conducted to assess the suitability of enriched *U. rigida* as a feed for *Haliotis roei* and to compare growth rates of this diet with growth rates on various artificial diets.

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MATERIALS AND METHODS

H. roei were collected from limestone reef platforms in the Perth metropolitan area in March 1999. Abalone were gently removed from the reef top and immediately placed into aerated seawater for transport. All animals were tagged (Hallprint Pty Ltd, FPN shellfish tags), and then randomly distributed into 28 trial tanks after being weighed to the nearest 0.01 g (whole wet body weight; WWBW), and measured with manual calipers (0.1 mm accuracy). The initial mean length and weight of the abalone were 31.9 ± 4.3 mm and 6.17 ± 2.28 g (mean ± SE) ($n = 560$).

Trial System

Abalone were kept in rectangular 27-L plastic aquaria with artificial grass strips above the water line to prevent abalone from climbing out of the tanks. Twenty abalone were allocated to each trial tank (four replicates per diet), and all 28 tanks were arranged randomly in relation to experimental diet. The system was flowthrough and used a seawater bore as the water source. All tanks were individually aerated. The flow rate to each tank was maintained at 1 L min^{-1} and checked weekly. Water quality parameters (dissolved oxygen [DO], pH, and temperature) were randomly measured during each feed with systematic analyses made in each tank weekly.

Diets

Diets used in this trial are listed in Table 1. All artificial diets were commercially available (with the exception of the FRDC2 control diet), and enriched *U. rigida* was grown in high nutrient water (5 g N m^{-2} day^{-1}; 0.6 g P m^{-2} day^{-1}) on inorganic nutrients (NH_4Cl and Na_2HPO_4) in accordance with Shpigel et al. (1999). The abalone were fed 3% bw/day, which is in excess of requirements for greenlip abalone, *H. laevigata* (Tom Coote 1999 pers. comm.). Tanks were cleaned by siphon every second day. Feed rates for *U. rigida* were calculated on a dry weight basis. This was based on enriched *U. rigida* possessing a water content of 84%. The control diet, known as the FRDC2 formulation, was made available by the Fisheries Research and Development Council

TABLE 1.

Artificial diets used.

Treatment	Diet	Protein Content (% Dry Weight)
1	FRDC 2—*Authors*	35
2	AAFDG—*Adam and Amos*	27
3	Enriched *Ulva rigida*	32.2
4	Abfeed—*Sea Plant Products*	34.6
5	Haliogro—*E.N. Hutchinson*	30.0
6	Deakin Diet—*Marine Feeds*	——
7	Diet 7—confidential	——

Note: feed manufacturers are listed in italics.

(FRDC). The dry ingredients included fishmeal, soyflour, semolina, sodium alginate, and $CaCO_3$. The Deakin diet (Diet 6), and Adam and Amos (Diet 2) diet, were both commercially available within Australia. Haliogro (Diet 5) was from New Zealand, and AB-feed (Diet 4) was from South Africa. It should be noted that none of the diets used in this trial was specifically formulated for *H. roei*.

Analysis of U. rigida

Proximate analysis of *U. rigida* was undertaken throughout the trial to quantify moisture, ash, protein (nitrogen × 6.25), fat, and carbohydrate levels. Moisture content was determined by gravimetric weight loss on heating the samples to a constant weight at 105°C (Association of Official Analytical Chemists [AOAC] method number 950.46B). Ash was determined by gravimetric weight loss on ashing the samples in a muffle furnace at 550°C (AOAC method number 920.153). Analysis of nitrogen content utilized the Kjeldahl method (AOAC method number 928.08), and fat/lipid levels were determined via acid hydrolysis and solvent extraction (AOAC method number 950.54).

Consumption Analyses

Consumption was assessed twice during the trial by manually siphoning uneaten feed from tanks. Consumption was estimated by relating the dry weight of the uneaten food to the known dry weight of the feed provided. Consumption data were corrected for dry matter weight loss attributable to leaching by leaching all diets over a 48-hr period and drying to a constant weight. Consumption estimates involved the collection of all uneaten food before cleaning on the second day of the feed/clean cycle, through manual siphon onto 100-μm mesh screen. Collected feed was carefully washed onto a circular filter mesh for drying and weighing. Feed was dried at 55–60° C for a period of 2 days before weighing.

Salinity Stress Test

A 96-h stress test for over-all health was conducted to assess the effect of diet on abalone health. Salinities for the stress test were derived from a preliminary assessment of salinity tolerance for *H. roei* and the limited literature regarding salinity tolerances of abalone (Nakanishi 1978, Mgaya & Mercer 1994, Jarayabhand & Paphavasit 1996, Boarder & Maguire 1998). Abalone were held in PVC cages (see Harris et al. 1997) and acclimatized in full strength seawater for 4 days (Boarder & Maguire 1998). Twelve abalone were randomly assigned to each cage. Upon acclimation, one cage from each diet was suspended vertically within an aerated 1,000-L tank of a particular salinity. Salinities used were 35, 25, and 20‰, and each salinity was replicated twice. Cages were checked every 4 h for 96 h by lifting the cage clear of the water and gently shaking. Abalone no longer attached to the cage and nonresponsive to touch on the mantle were classified as mortalities. Animals responsive to touch and not attached to the cage were recorded as detachments and returned to the cages.

RESULTS

Growth

Specific growth rate (SGR) was significantly affected by diet, as measured by either whole weight ($P < 0.05$) or by shell length ($P < 0.01$). Growth rates for *H. roei* fed inorganically enriched *U. rigida* (D3) were not significantly different (Tukey's test, $P > 0.05$) to growth achieved from the best performing artificial diets. The abalone grew best on the Adam and Amos (D2) diet for both length and weight (SGR-L = 0.161 ± 0.004; SGR-W = 0.495 ± 0.2). However, growth was not significantly higher than on the FRDC2 (SGR-L = 0.139 ± 0.006; SGR-W = 0.371 ± 0.035), *U. rigida* (SGR-L = 0.142 ± 0.010; SGR-W = 0.379 ± 0.040), or AB-feed (SGR-L = 0.131 ± 0.006; SGR-W = 0.4113 ± 0.014) diets. This is shown in Figures 1 and 2, respectively.

Consumption Analyses

Consumption rate was significantly affected ($P < 0.01$) by diet for both consumption assessments. The two consumption analyses were also significantly different from each other ($P < 0.01$), as shown in Figure 3. Diets 2, 5, 6, and 7 were all observed to be less water stable than Diets 1, 3, and 4 after 2 days within the treatment tanks.

Salinity Stress Test

Abalone held in full strength seawater and 25‰ salinity exhibited 100% survival at the conclusion of the 96-h trial, with the exception of abalone fed on Diet 3, which exhibited 92% survival over the same period in 25‰. Detachment rates for both of these salinities were negligible for all diets at the higher salinities.

Diet significantly affected survival at 20‰ ($P < 0.05$). No abalone fed Diet 3 (enriched *U. rigida*) survived in 20‰ after 96-h (Fig. 4). Survival was significantly the same for all other diets (Tukey's test $P > 0.05$), with an average survival of 40.28 ± 4.33%.

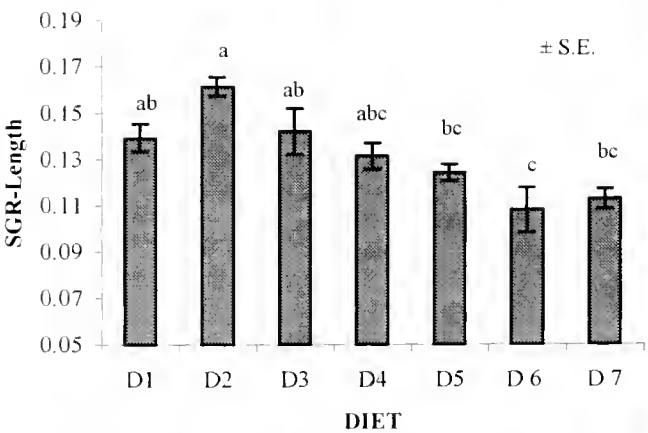

Figure 1. Effect of diet on specific growth rate for length (mm) for juvenile *Haliotis roei* ($n = 4$). *Note*: Diets sharing the same letters are not significantly different (Tukey test, $P > 0.05$).

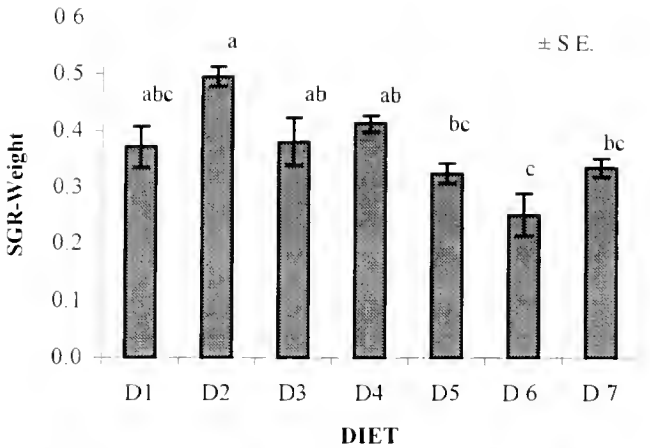

Figure 2. Effect of diet on specific growth rate for weight (g) for juvenile *Haliotis roei* (n = 4). *Note*: Diets sharing the same letters are not significantly different (Tukey test, P > 0.05).

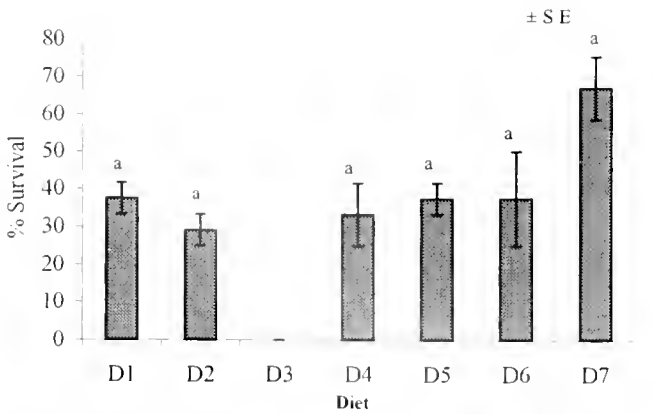

Figure 4. Effect of diet on survival after 96 h at 20% salinity for juvenile *H. roei* (n = 2; 12 abalone per replicate). *Note*: Diets sharing the same letters are not significantly different (Tukey test, P > 0.05).

Diet 3 was not included in the analysis of variance (ANOVA) because of zero variance caused by 0% survival. Mortality on Diet 3 was also observed earlier than all other diets (Table 2). Fifty percent of all animals fed enriched *U. rigida* died after 68 h, as compared with an average of 9.73 ± 2.06% for abalone fed other diets over the same time period. Most other diets reached 50% mortality after approximately 85 h, almost 20 h later than the *U. rigida*-fed abalone (Table 2). Abalone fed Diet 3 were also observed to detach earlier than those on other diets.

DISCUSSION

Growth rates of *H. roei* fed enriched *U. rigida* were not significantly different from growth rates achieved on the three best performing artificial diets ($P > 0.05$). Previous growth trials with *Ulva* spp. as a feed for abalone species have used "wild" *Ulva* spp. or *Ulva* spp. that had not been enriched (Mai et al. 1994, Mai et al. 1996, Simpson & Cook 1998). These growth trials have shown the effectiveness of *U. rigida* as a feed to be totally dependent on the abalone species to which it is being fed. Mai et al. (1996) found that although wild *U. lactuca* was a "moderately good diet" for *H. tuberculata*, it was the worst of five diets for *H. discus hannai*. Corazani and Illanes (1998) showed that *H. discus hannai* grew significantly faster on *U. rigida* than on other macroalgal diets, and the authors recommend juvenile abalone of this species be fed a diet of *Ulva* spp. in combination with an artificial diet. They felt that the artificial component of the diet was a supplement to the *Ulva* spp. and should be fed at levels no higher than 1% of total body weight day^{-1}.

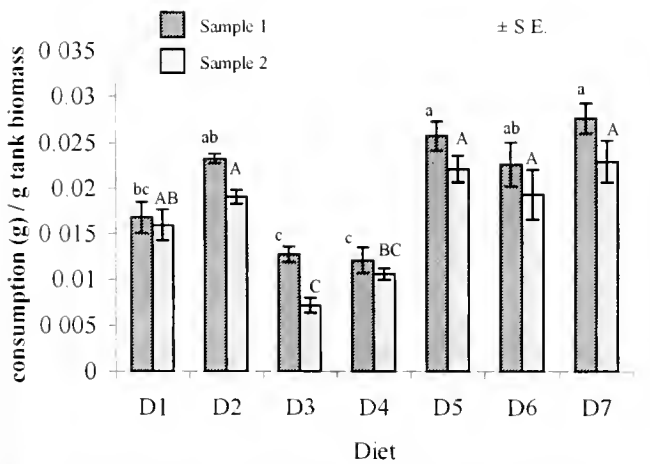

Figure 3. Effect of diet on consumption rate (grams dry feed consumed/gram tank biomass) for juvenile *Haliotis roei* at two separate sampling times (n = 4). *Note*: Diets sharing the same letters are not significantly different (Tukey test, P > 0.05).

Simpson and Cook (1998) investigated the growth of *H. midae* grown on various natural algal diets. They found that after 4 mo on trial diets, abalone fed on a diet of wild *Ulva* sp. were significantly shorter in shell length than abalone fed on most other macroalgal diets. *Ulva*-fed animals were also found to have the lowest wet weight to shell length ratios. However, when the animals were fed *Ulva* as a proportion of a mixed diet, they exhibited excellent growth rates, suggesting that *Ulva* provided essential nutrients not found in the other algae fed.

The increase in protein content of *U. rigida* observed within this trial is supported by previous research in which *Ulva* spp. have been used as macroalgal biofilters (Tenore 1976, Neori 1996, Shpigel et al. 1996a, Shpigel et al. 1996b). In the current trial, wild *U. rigida* was found to have a protein content of 13% (% dry weight), compared with 32.2 ± 1.5% (% dry weight) for *U. rigida*, which had been enriched on inorganic nutrients. The high protein content throughout the trial could explain the differences observed between the growth achieved on wild *Ulva* spp. in other research and the growth achieved within this trial *U. rigida*. Fleming (1995) found that the intake of digestible nitrogen directly influences the growth rates of *H. rubra*. This suggests that nitrogen may be a limiting factor for growth in *Haliotis* spp. Britz and Hecht (1997) support this view by stating that maximum growth can only be achieved when sufficient protein, in the correct proportions of amino acids, is supplied in the feed. Shpigel et al. (1996a; 1999) state that the good growth of *H. tuberculata* and *H. fulgens* when fed enriched *U. lactuca* was attributable to a consistent supply of high protein diet.

The consumption results in this study do not adequately reflect the trends observed within the tanks. Some diets were observed to be more water stable than others, and thus were easier to fragment upon siphoning for collection. Diets 1 and 4 (FRDC2 and

TABLE 2.

Effects of diet on time (hours ± SE) taken to reach 50% mortality for Juvenile *H. roei* held at 20‰ saline (n = 2; 12 abalone per replicate).

Diet	1	2	3	4	5	6	7
LS_{50} Time @ 20‰	84 ± 4^{ab}	86 ± 2^{a}	68 ± 4^{b}	82 ± 2^{b}	88 ± 4^{a}	86 ± 2^{a}	>96

Note: Diets sharing the same letters are not significantly different ($P > 0.05$).

AB-feed) were both extremely water stable within the tank and, thus, held together well through the siphoning collection process. Diets 2, 5, and 6 (Adam & Amos, Haliogro and Deakin) were less water stable, but were still firm after 2 days. These diets fragmented when they were siphoned, as did Diet 7, which was very soft after 2 days within the tanks. The data were potentially biased against Diets 1, 3, and 4 because of the fragmentation of other diets causing some loss of material during analysis. This would have reduced the amount of feed actually collected; thereby, increasing the apparent consumption. Differences in the various diets' water stability may be attributable to the diets having being formulated for colder water species.

Stress determination for aquatic animals usually involves a quantitative evaluation of a variable that is directly affected by the stressor. The application of an environmental stress allows for an assessment of general health or vigor for animals from different treatments. Salinity stress tests have been used previously to determine the quality of prawn post larvae (Briggs 1992, Samocha et al. 1998), fish larvae (Dhert et al. 1990a, Dhert et al. 1990b) and the effects of diet on abalone robustness (Boarder & Maguire 1998). Boarder and Maguire (1998) found that dietary vitamin levels directly determined the survival of *H. laevigata* at 23‰ salinity. Animals fed on twice the normal dietary inclusion level of vitamin mix exhibited over 90% survival after 96 hours, as compared with approximately 50% survival for animals fed the normal dietary vitamin level.

The poor survival at 20‰ for abalone fed *U. rigida* within this trial may be attributable to the osmoregulatory mechanisms utilized by *Haliotis* spp. rather than a general lack of health. Abalone species are known ionic conformers; therefore, blood osmolality and ionic concentration closely resemble those of the external environment (Somero & Bowlus 1983). The ability to regulate cell volume is also an important ability in soft-bodied osmoconforming marine animals (Tarr 1976, Burton 1983), because this prevents cells from rupturing upon exposure to low salinities. Cell volume regulation within some osmoconforming mollusks is controlled by organic solutes, such as amino acids (Burton 1983, Mai et al. 1994). At low salinities, amino acids are excreted from the cell along with osmotically obligated water, thereby restoring cell volume (Pierce & Amende 1981). Pierce and Amende state that there is a possibility that the physiological response of osmoconforming mollusks to low salinity may be directly related to their ability to maintain and control a large intracellular free amino acid pool.

The most important free amino acids for cell volume regulation (dependant on abalone species) are taurine, glycine, alanine, and proline, with an emphasis on taurine (Burton 1983). Biosynthesis of the amino acid taurine from methionine is known to occur in gastropod mollusks (Mai et al. 1994). Mai et al. (1994) found that the levels of taurine in the viscera of *H. discus hannai* were significantly lower in abalone fed wild *U. lactuca* than abalone fed on all other diets. The authors also found that the viscera methionine levels within the abalone fed *U. lactuca* were extremely high, indicating some physiological inhibition in the biosynthesis of taurine from methionine. Toxic substances contained within *U. lactuca*, as detailed by Borowsky and Borowsky (1990), were thought to cause this inhibition. Johnson and Welsh (1985) found that an exudate excreted from *U. lactuca* killed 100% of estuarine crab larvae within 24 h. The authors also report that the bactericidal and fungicidal properties of *Ulva* spp. exudate have been known for some time.

Because taurine is one of the most important amino acids for cell volume regulation, inhibition of taurine production from methionine could significantly affect the cell volume regulation ability of abalone fed solely on *Ulva* spp. The comparatively good growth observed in *H. roei* fed *U. rigida* within this trial indicates that there was no feeding inhibition caused by antinutritional compounds or toxic substances contained within the seaweed. Fleming (1995) found that antinutritional compounds within some algal species did not prevent preferences for algae containing high levels of digestible nitrogen. This suggests that, although the abalone fed on enriched *U. rigida* were capable of good growth, they were not capable of adequate cell volume regulation when exposed to hypo-osmotic conditions because of a lack of taurine within their free amino acid pool. It is important to note that all other health parameters (including growth) were excellent for abalone fed solely on enriched *U. rigida*.

The results of this trial indicate the salinity tolerance of *H. roei* to be between 25 and 20‰. This correlates closely with the limited literature on *Haliotid* salinity tolerances, which indicate short-term survival is possible at salinities around 20‰ (Singharaiwan et al. 1992, Jarayabhand & Phapavisit 1996, T. McCormick 1997 pers. comm., Boarder & Maguire 1998). The 96-h LS50, the salinity at which 50% of the test animals survive after 96 h, is relatively close to 20‰ for *H. roei*. Most diets displayed 50% survival at just under 96 h within this trial. This compares with only 10% survival after 96 h for *H. diversicolor supertexta* juveniles transferred from 35 to 20‰ at similar temperature (Chen & Chen 2000).

In conclusion, enriched *U. rigida* is a suitable feed for *H. roei*, producing comparable growth rates to several commercially available manufactured feeds. Diet directly affects survival of abalone under hypo-osmotic stress, and *U. rigida* may impair the ability to cope with this stress by affecting cell volume regulatory mechanisms.

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SEASONAL ENERGETICS OF *HALIOTIS FULGENS* (PHILIPPI) AND *HALIOTIS TUBERCULATA* (L.)

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ABSTRACT Bioenergetics (food ingestion, absorption, growth, respiration, ammonia, and mucus production) of the warm-water abalone *Haliotis fulgens* and the cold-water species *H. tuberculata* were studied under summer and winter conditions in the Gulf of Aqaba. Individuals of 38–45 mm shell length and 7–11 g whole live weight were fed *Ulva lactuca* and *Gracilaria conferta*. Food ingestion was greater in summer but not sufficient to overcome the high maintenance, resulting in low (*H. fulgens*) or no (*H. tuberculata*) growth in summer. Growth for *H. fulgens* and *H. tuberculata* during winter was 0.10 ± 0.05 mm/d and 0.09 ± 0.02 mm/d and 0.02 ± 0.01 and 0.03 ± 0.01 g DW/d, respectively. Respiration rate and ammonia excretion were greater in summer for both species. In summer, *H. tuberculata* had greater food absorption than *H. fulgens*, and the reverse was found in winter. Mucus secretion was only 1–9% of the energy budget. In winter, growth accounted for the majority of the energy budget of both species and in summer, respiration and ammonia were the main components. Overall, *H. fulgens* seems to be most suited to year-round conditions found in the Gulf of Aqaba.

KEY WORDS: abalone, bioenergetics, *Haliotis fulgens*, *Haliotis tuberculata*

INTRODUCTION

Nonindigenous species must have certain characteristics if they are to be successfully cultured in a new environment. In particular, candidates for aquaculture must be acclimated and problems identified quickly. It is essential to know the degree of compensation possible to environmental change and to understand how they function in their natural environment. An energy budget relates the growth of an animal to its physiological condition and is one way to understand the basic biology of an animal and observe its energy partitioning for maintenance and growth in the new environment.

Previous research has shown that *Haliotis fulgens* and *H. tuberculata* are suitable for culture in warm-water environments (Shpigel et al. 1996a, Shpigel 1996b). However, comparison of the performance of these two species and description of the process by which ingested food is utilized for maintenance and growth is not known for warm seawater conditions. Growth and production characteristics can be measured using energy budget parameters. Food consumption, feces egestion, respiration, ammonia excretion, and mucus secretion are essential elements in an abalone energy budget (Peck et al. 1987, Barkai & Griffiths 1988, Donovan et al. 1998). Nutrient relationships are also useful in understanding an energy budget and indicate the role of digestive characteristics in energy utilization. Food absorption and food conversion efficiencies have been used in other invertebrates to indicate interactions between metabolism and production (Lawrence 1975, Bayne et al. 1975).

H. fulgens is a warm-water species found in seawater of 12–23°C (Hahn 1989) but tolerates temperatures up to 28°C for short periods (Leighton et al. 1981). *H. tuberculata* is a cold-water species found in seawater of 5–18°C (Mgaya 1995) but shows accelerated growth when cultured in seawater near 20°C (Koike et al. 1979, Shpigel et al. 1996a). *H. fulgens* and *H. tuberculata* are benthic, motile to semisessile subtidal species relying on drift and attached macroalgal communities for food. The objective of this study was to determine the status of *H. fulgens* and *H. tuberculata* by measuring energy budget parameters in summer and winter.

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METHODS

Two species of abalone, *Haliotis fulgens* and *H. tuberculata*, were studied for two seasons, winter and summer. Individuals of 38–45 mm shell length and 7–11 g whole live weight were selected from cultured populations at the National Center for Mariculture in Eilat, Israel. Incoming seawater was filtered by sand and cartridge filtration to 1 μ. Seawater temperatures were 24–28°C and 20–22°C in summer and winter, respectively. Dissolved oxygen was always near saturation, and salinity was 41 ± 1 ppt. Ambient photoperiod was 14 h of light in summer and 11 h of light in winter. Animals were held in glass aquaria of 25 L. For each species, five replicate aquaria held 10 individuals per season. Abalone were selected randomly from two aquaria for respiration, ammonia, feces, and mucus measurements. Individuals were used only once for metabolic measurements. Three aquaria per species were used to determine feeding and growth rates. These individuals were not used for physiologic measurements. A preliminary energy budget including ingestion, assimilation, respiration, ammonia production, feces, mucus secretion, and growth was prepared showing the allocation of energy in joule/g DW/day in summer and winter.

Somatic Growth (G)

H. fulgens and *H. tuberculata* were measured at the start and finish of each seasons' experiments to determine daily growth increments. Growth was measured as the change in shell length (mm) and whole dry weight (g) during the summer interval, 7 July to 5 October 1995, 93 days, and the winter interval 12 December 1995 to 5 March 1996, 83 days. Specific growth rate was calculated for weight (g) and length (mm) as [(*ln* mean final abalone dry weight (length) − *ln* mean initial abalone dry weight (length))/days per experimental period] * 100. Growth as change in energy content (joule/g DW/day) was calculated for the energy budget using the dry tissue weight of the abalone.

At the beginning of the study, samples of each *H. fulgens* and *H. tuberculata* were weighed to the nearest 0.001 g, measured to 0.01 mm, and dried at 60°C for 7 days to determine whole wet weight/dry weight ratio of the abalone. The dry tissue/dry shell ratio was calculated after drying. The dried abalone tissue was

combusted in a Parr semimicro bomb calorimeter using benzoic acid as a standard to determine gross energy content (joule/g DW).

Consumption (C)

The abalone were fed *Ulva lactuca* and *Gracilaria conferta ad libitum* at a ratio of 3:1. Food introduced to the abalone and uneaten food removed was blotted on paper toweling to remove excess moisture. Dry food ingestion was measured over 1-wk intervals four times each season. Algae held in aquaria without abalone had no significant weight change. Feed ingestion was calculated directly as the difference between algae added and removed from the aquaria.

The protein content, dry and ash weight of the algae were measured each season. Dry and ash weights were determined by drying to a constant weight at 60° C followed by incineration for 24 h at 550°C. Crude protein was measured using the Kjedahl method and multiplying *n* by 6.25. The gross energy content (cal/g DW) of *U. lactuca* and *G. conferta* was determined by combustion in a semimicro bomb calorimeter using benzoic acid as a standard.

Gross food conversion efficiency was calculated using the food ingested in terms of energy as (mean final joule/g DW–mean initial joule/g DW)/ (mean joule/g DW food consumed/abalone/season) × 100. The dry matter content of the abalone tissue was used for food conversion efficiency. Net food conversion efficiency for joule/g DW/day was calculated using the food absorbed.

Feces (F) and Absorption Efficiency

Feces were collected by placing individual abalone in a 1 L conical tank with a 1 mm mesh floor. The water containing the feces was filtered onto the dried, preweighed GFC filters, dried at 60°C for 24 h. Dry feces were incinerated for 3 h at 450°C in muffle furnace. The dry and ash weights were used to calculate the organic content of the feces.

The absorption efficiency was calculated by the indirect method of Conover (1966): $U' = F' - E'/(1-E')(F')$, where U′ is the absorption efficiency, and F′ and E′ are the concentrations of organic matter in the food and feces, respectively.

The energy expended in feces egestion was calculated from the food absorption values. The gross energy values of *H. tuberculata* feces, 2,817 cal/g dry weight, determined by Peck et al. (1987) were used for our energy budget calculations.

Respiration (R)

A 720-mL round, Perspex respiratory chamber with a magnetic stirrer submersed in a flowthrough seawater bath was used to measure oxygen levels every minute until 50% oxygen saturation was reached. Test Point® software was used to measure the incoming mg O_2/L with an accuracy of 0.01 mg O_2/L. Each respiration determination lasted for approximately 3 h. Measurements were made during the day and night. Results are presented per 24 h. Five chambers were run concurrently. Respiration measurements were made over 2-wk intervals each season. Night respiration was completed between 2300 to 0200 h. Day respiration was completed between 1100 to 1400 h. For the energy budget, the daily oxygen consumption, mg O_2/g DW/day was calculated from the day and night rates. Oxygen consumption was converted to energy using 3.47 cal/mg O_2 (Bayne et al. 1975).

Ammonia (U)

In separate tests, individual abalone were placed in respiration chambers. Seawater collected from the chambers was analyzed with a Technicon II Autoanalyzer giving μM NH_4^+/h/abalone. Ammonia was analyzed following filtration through HCl washed Whatman GF/C filters according to the modified oxidation procedure of Neori et al. (1996). Three 1-mL aliquots were collected from each abalone. For the energy budget, a mean daily ammonia excretion rate μM NH_4^+/g DW/day was calculated for day and night. Conversion factors used to calculate energy equivalents of ammonia excretion were μ18 M ammonia/μg ammonia and 0.0249 joule/μg ammonia (Bayne et al. 1975). Samples were collected during the night and day as for respiration measurements. Each test lasted 2.8 to 3.5 h.

Mucus (M)

Individual abalone were moved to clean aquaria without feed and with low aeration for 48 h. The abalone were removed, and the walls of the aquaria were cleaned with a spatula. The mucus from the aquaria water and walls were collected on preweighed, dried, GF/C filters. The filters were rinsed with distilled, deionized water to remove salts, and dried at 60° C. Mucus samples were pooled for all aquaria per species. The caloric value of mucus used was 5,439 cal/g dry weight (Peck et al. 1987).

Energy Budget

An energy budget was calculated for *H. fulgens* and *H. tuberculata* during summer and winter based on joule/g DW abalone/day using food ingestion (C), somatic growth (G), respiration rate (R), ammonia excretion (U), feces egestion (F), and mucus secretion (M) as parameters. The scope for growth, all maintenance energy (respiration, ammonia, mucus, and feces) subtracted from the total absorbed energy (A) (Widdows et al. 1989) was also calculated.

Statistics

Somatic growth, respiration, ammonia production, dry feed ingestion, organic content of the feces, and total organic absorption were compared between species and between seasons by Student's *t*-test. All mucus samples were pooled to give one value per species per season. Specific growth rates for dry weight and shell length were calculated from means resulting in one value per species per season.

RESULTS

The whole wet weight (g)/whole dry weight ratio for *H. fulgens* and *H. tuberculata* are 0.47 ± 0.3 and 0.43 ± 0.2, respectively (n = 20 in each sample). The dry tissue/dry shell ratio is 0.36 ± 0.07 and 0.33 ± 0.3 for *H. fulgens* and *H. tuberculata*. The caloric content of dry tissue is 4,848 cal/g DW and 4,600 for *H. fulgens* and *H. tuberculata*, respectively.

Somatic Growth (G)

In summer, *Haliotis fulgens* had a greater increase in shell length than *H. tuberculata,* but there were no significant differences (Table 1). At the end of the summer interval, *H. tuberculata* lost weight as compared to *H. fulgens*, which showed a slight weight gain. The mean weight loss of *H. tuberculata* resulted in significant differences between species in this measurement of somatic growth. In winter, shell length and dry weight increase were significantly greater for *H. tuberculata* than *H. fulgens*. Somatic growth was greater for both species in winter than summer (Table 2). The specific growth rates (SGR), daily changes in DW, and shell length reflected the growth pattern seen from direct

TABLE 1.

Student's *t*-test results of somatic growth, respiration, ammonia, and mucus production of *Haliotis fulgens* and *H. tuberculata* compared between species.

Growth	Season	Species (mean ± SD) *H. fulgens*	Species (mean ± SD) *H. tuberculata*	Test statistic (t)	df	*P*	Significance
Shell length mm/day	Summer	0.02 ± 0.02	0.01 ± 0.01	1.094	7	0.695	NS
	Winter	0.10 ± 0.05	0.09 ± 0.02	0.397	7	0.291	NS
Dry weight g/day	Summer	0.01 ± 0.001	−0.01 ± 0.01	3.896	7	0.001	**
	Winter	0.02 ± 0.01	0.03 ± 0.01	−2.847	7	0.001	**
Feed dry g/day	Summer	0.02 ± 0.01	0.04 ± 0.01	−5.233	7	0.002	*
	Winter	0.02 ± 0.01	0.03 ± 0.01	−5.509	7	0.002	
E′ (%)	Summer	33 ± 2	22 ± 1	5.711	7	0.001	*
	Winter	30 ± 2	44 ± 2	−4.102	7	0.001	
U′ (%)	Summer	76 ± 2	87 ± 5	−5.149	7	0.002	*
	Winter	80 ± 3	64 ± 3	4.065	7	0.001	*
mg O_2/g DW/d	Summer	3.9 ± 1.3	11.1 ± 4.5	−3.466	8	0.008	
	Winter	2.1 ± 0.9	2.3 ± 0.9	−0.545	12	0.596	NS
µg NH_4+/g DW/d	Summer	17.1 ± 0.6	67.2 ± 32.0	−3.837	6	0.009	
	Winter	14.0 ± 5.6	15.5 ± 6.5	−0.361	7	0.728	NS

(* $P < 0.05$; ** $P < 0.001$; NS not significant; g DW = g dry weight; E′ organic content of the feces, U′ organic matter absorbed).

growth measurements (Table 3). Energy used for growth was greater in winter than in summer.

Consumption (C)

Dry feed ingested/g DW abalone/day was significantly greater for *H. tuberculata* than *H. fulgens* during both seasons (Table 1). Neither species had significantly different feed ingestion rates when compared between seasons (Table 2). Gross food conversion efficiency was greatest for *H. tuberculata* in the winter, followed by *H. fulgens* in the winter, *H. fulgens* in the summer, and least efficient in *H. tuberculata* during summer months (Table 3).

Algal protein content was greater in winter than summer ($t = -2.5052$, df = 12, $P = 0.028$, $t = -8.483$, df = 12, $P = 0.005$) for *Ulva lactuca* and *Gracilaria conferta*, respectively. Caloric values were not significantly different between seasons (Table 4).

Organic absorption was significantly different between species in both seasons. *H. tuberculata* had a higher food absorption rate than *H. fulgens* during summer, and the reverse was true in winter. The organic content of both algal species was the same, 70%, and did not change with season (Table 2). Food absorption in *H. tuberculata* in winter was the lowest found in this study.

Feces (F)

The organic content of the feces was significantly greater for *H. fulgens* in winter and for *H. tuberculata* in summer. The organic content of the feces was significantly greater for *H. tuberculata* in winter than summer. Organic content of the feces had an inverse relationship with the organic absorption (Tables 1 and 2).

Respiration (R)

H. fulgens and *H. tuberculata* had greater respiration rates in summer than winter. There were no significant differences in win-

TABLE 2.

Student's *t*-test results of somatic growth, respiration, ammonia, and mucus production of *Haliotis fulgens* and *H. tuberculata* compared within species between seasons.

	Species	Test statistic (*t*)	DF	*P*	Significance
Shell length (mm/day)	*H. fulgens*	−4.465	7	<0.001	**
	H. tuberculata	−9.116	7	<0.001	**
Dry weight (g/day)	*H. fulgens*	−4.196	7	<0.001	**
	H. tuberculata	−6.230	7	<0.001	**
Feed dry g/day	*H. fulgens*	−1.276	7	0.246	NS
	H. tuberculata	−1.066	7	0.327	NS
E′ (%)	*H. fulgens*	0.756	7	0.464	NS
	H. tuberculata	−8.341	7	<0.001	**
U′ (%)	*H. fulgens*	−1.059	7	0.312	NS
	H. tuberculata	5.677	7	<0.001	**
mg O_2/g DW/24 h	*H. fulgens*	3.030	11	0.011	*
	H. tuberculata	4.687	9	0.001	**
µg NH_4+/g DW/24 h	*H. fulgens*	0.140	8	0.892	NS
	H. tuberculata	3.236	5	0.023	*

* $P < 0.05$; ** $P < 0.001$; NS not significant; g DW = g dry weight; E′ organic content of the feces; U′ organic matter absorbed.

TABLE 3.

Specific growth rates as % DW/d and % SL/d and food conversion efficiencies (FCE) for *Haliotis fulgens* and *H. tuberculata*.

	H. fulgens		*H. tuberculata*	
	Summer	Winter	Summer	Winter
SGR % DW/d	0.19	0.29	−0.07	0.63
SGR % SL/d	0.03	0.06	0.01	0.22
Gross FCE—%, joule/g DW/d	6.6	9.0	−0.5	15.5
Net FEC—%, joule/g DW/day	8.7	11.2	−0.6	24.2

TABLE 4.

Energy and protein content (mean ± SD) of *Ulva lactuca* and *Gracilaria conferta*.

	Ulva lactuca		*Gracilaria conferta*	
	Summer	Winter	Summer	Winter
Gross energy (cal/g DW)	3278 ± 166	3299 ± 191	3657 ± 59	3339 ± 170
Crude protein (%/DW)	13.3 ± 2.2	24.2 ± 5.9	20.9 ± 4.6	29.3 ± 2.8

TABLE 5.

Day and night respiration values (mgO_2/g DW/h, mean ± SD) for *Haliotis fulgens* and *H. tuberculata*.

	H. fulgens		*H. tuberculata*	
	Day	Night	Day	Night
Summer	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.2
Winter	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1

TABLE 6.

Day and night ammonia excretion values (μMHN_4^+/g DW/h, mean ± SD) for *Haliotis fulgens* and *H. tuberculata*.

	H. fulgens		*H. tuberculata*	
	Day	Night	Day	Night
Summer	0.3 ± 0.1	1.0 ± 0.3	1.3 ± 0.3	5.3 ± 3.1
Winter	0.6 ± 0.2	0.7 ± 0.2	0.6 ± 0.3	0.7 ± 0.4

TABLE 7.

Energy budget per food ingested and scope for growth per energy absorbed; units are joule/g DW/d unless otherwise indicated.

	H. fulgens		*H. tuberculata*	
	Summer	Winter	Summer	Winter
C	274	248	509	427
A	207	438	198	273
R	56	30	161	34
U	32	26	125	29
F	58	50	68	154
M	23	17	8	6
G	51	−13	116	164
Other	45	14	156	41
% of total C	17	6	30	10
Scope for growth	30	49	−13	154

ter, but *H. tuberculata* had a greater rate than *H. fulgens* in summer. For both species, respiration was similar for day and night measurements, except for *H. tuberculata* in the summer, where night respiration was greater (Table 5).

Ammonia (U)

Ammonia excretion rate was significantly greater for *H. tuberculata* than *H. fulgens* in summer. There were no significant differences between species in winter. When compared between seasons, *H. tuberculata* had a greater rate in summer than winter, and there were no significant differences in the ammonia excretion of *H. fulgens* between seasons (Tables 1, 2).

Ammonia excretion was similar during day and night measurements, except for *H. tuberculata,* which showed a higher rate during the summer night samples (Table 6).

Mucus (M)

Mucus collected from all aquaria per species per season was pooled. Mucus secretion was 0.004, 0.001, 0.003, and 0.001 g DW/abalone/day for *H. fulgens*-summer, *H. tuberculata*-summer, *H. fulgens*-winter, and *H. tuberculata*-winter, respectively.

Energy Budget

For both species and seasons, 83–94% of the assimilated energy was accounted for, except *H. tuberculata* in the summer, where 70% of the total energy intake was measured (Fig. 1). Because of higher feed ingestion rates in summer, total energy ingested was greater than in winter. In the summer, *H. tuberculata* responded to warm seawater conditions by high respiration rate and ammonia excretion. *H. fulgens* had the highest mucus secretion rate overall, particularly during summer.

The scope for growth was greater in winter than summer. Although *H. tuberculata* would have been expected to show growth rates similar to *H. fulgens* in the summer by the scope for growth calculation, this species lost weight and showed a slight increase in shell length.

DISCUSSION

The energy budget published for *H. tuberculata* (Peck et al. 1987) was done in its normal range and offers valuable insight to our results. *H. fulgens* has very little basic biological information published, thus we were unable to make any direct comparisons of metabolic and nutritive characteristics.

In general, the food ingestion increased in summer for both species. As expected, the energy expenditures in body processes of *H. fulgens* and *H. tuberculata* also intensified during the summer. Additional energy was available from the food ingested, but most of this was used for maintenance. The significant difference in responses of *H. fulgens* and *H. tuberculata* may result from the seawater temperatures tested here: 20–26°C.

H. fulgens is a relatively warm-water species and has shown accelerated growth for short periods at 28°C (Leighton 1981). *H. tuberculata* has maximal growth at temperatures near 20°C (Mgaya 1995, Shpigel et al. 1996a) and negative growth at 26°C (Shpigel et al. 1996b). On an annual basis, *H. fulgens* had greater growth in shell length (22.1 vs. 18.5 mm/y), and *H. tuberculata* has greater growth in whole dry weight (10.6 vs. 9.0 g/y). The growth rates found here may not represent maximal performance of *H. tuberculata,* because higher growth rates were found between

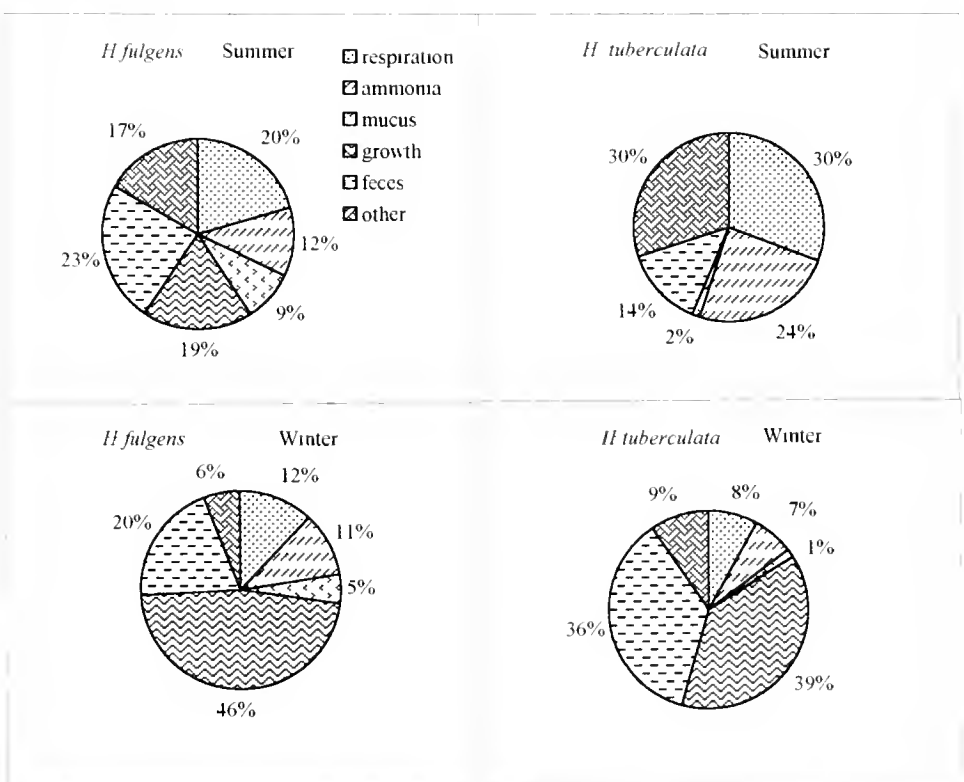

Figure 1. Energy budgets for *Haliotis fulgens* and *H. tuberculata* for summer and winter. Values are percentage of total energy ingested; other is energy not accounted for.

March and May (Neori et al. 1998), a time of year we did not study.

Fuller understanding of growth of these two species in a warm-water environment is shown in the scope for growth. The negative summer values indicate stress and reflect the high use of body reserves used for maintenance metabolism in both species. Other evidence of stress in *H. tuberculata* is demonstrated by the negative growth found in the energy budget. In contrast, *H. fulgens*, continued growing at a lower rate in the higher seawater temperatures recorded in summer months.

Mortalities observed during the decrease in seawater temperatures (Shpigel et al. 1996b) may result from the stressed condition of the *H. tuberculata* at this time. *H. tuberculata* may deplete stored body reserves of glycogen (Bennett & Nakada 1968) during summer. This combined with the additional stress of temperature change may explain these mortalities. The negative scope for growth found for *H. fulgens* may approach the lethal limit but seems less severe for this species.

Respiration is a useful and sensitive measure of daily energy expenditure. The stress of the higher seawater temperatures increased respiration rates. The solubility of oxygen changes little in the temperature and salinity range used in this study (Dejours 1988), but the ability of the abalone to cope with the seasonal environmental changes and maintain their metabolism was compromised. The higher rates of respiration relative to food intake found at temperatures between 24 and 26°C limited growth of both species. This relationship is also shown in the gross and net food conversion efficiencies that are lower for both species in summer.

The quality of the food is also important. Increased food ingestion in summer was probably more related to temperature than the slightly lower protein content of the algae in summer. The energy content may be more important in determining feed ingestion rates than protein (Shpigel et al. 1999). Energy content of *U. lactuca* and *G. conferta* was not significantly different between seasons.

The protein content of the algae fed here was consistent throughout the study and is higher than natural algal diets used in other studies. Cultured *U. lactuca* and *G. conferta* contains >20% protein, as compared to <11% found in natural populations (Peck et al. 1987, Barkai & Griffiths 1988, Shpigel et al. 1996b, Donovan et al. 1998). The elevated protein content seems to sustain growth in *H. fulgens* in summer and winter and provides adequate nutrition for significant growth of *H. tuberculata* in winter.

Organic absorption was significantly greater for *H. tuberculata* in winter than summer, again reflecting this species' ability for production in temperatures around 20°C. Seasonal variation in organic absorption is known for other invertebrates (McBride et al. 1998). The organic absorption of *H. fulgens* did not change significantly with season, again suggesting that this species is well adapted for production in seawater between 20–26°C.

The gross feed conversion efficiency indicates the efficiency with which the abalone utilizes the ingested energy for growth. Gross and net food conversion efficiencies were greatest for *H. tuberculata* during winter, because growth was high, and food ingestion and absorption were low, as compared to summer months, when growth was negative, and food ingestion was high. As with other measurements, the gross and net food conversion efficiency of *H. fulgens* were similar in summer and winter. The food conversion efficiencies were higher in winter for both species, showing more energy was available for growth and less was used for maintenance.

Mucus production was lower than other abalone energy budgets, between 1–9% of the total energy, as compared to 16–27% (Peck et al. 1987, Barkai & Griffiths 1988), except for *H. kamtschatkana* in winter, where mucus secretion contributed 4% of the total energy budget (Donovan et al. 1998). Two factors may contribute to the low mucus secretion values found here. First, *H. fulgens* and *H. tuberculata* move very little in culture condition at the National Center for Mariculture (pers. observ.). This is similar to the low activity found in *H. kamtschatkana* in winter (Donovan et al. 1998). Second, other studies determined mucus secretion by subtraction, and we calculated it by filtration. The combination of low activity of abalone in our study and our method of collection may have contributed to this difference

The scope for growth during winter months was similar for both species, reflecting their comparable growth rates. Although *H. tuberculata* lost weight during summer, their weight gain was greater on an annual basis than *H. fulgens*. Although not studied here, survival of *H. fulgens* seems to be greater than *H. tuberculata* because of seasonal seawater temperature changes (Shpigel et al. 1996b), adding more weight to the argument for pursuing culture of this species in a warm-water environment.

In conclusion, the energy budgets of *H. fulgens* and *H. tuberculata* provide a good indication of the animals' response to the culture environment. The mean seawater temperatures experienced by each species in its natural range strongly influenced expenditures of energy, resulting in low growth and high maintenance during summer and high growth and low maintenance in winter. In all factors measured, *H. fulgens* seems better adapted to the year-round culture conditions tested and would seem to be the most suitable species overall for culture in the Gulf of Aqaba.

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SPAWNING INDUCTION OF YELLOWFOOT ABALONE, *HALIOTIS AUSTRALIS* USING CHEMICALS AND GANGLIONIC SUSPENSIONS

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ABSTRACT A two-step experimental approach (*in vitro* and *in vivo*) using serotonin (5-hydroxytryptamine), dopamine (3-hydroxytyramine), prostaglandin E_2, deionized water, filtered seawater, cerebral (CG) and pleural-pedal (PPG) ganglionic suspensions as inducers of the spawning of *H. australis* were tested. A total of 88 laboratory conditioned abalone (n = 6–10 per treatment) with fully ripe gonads were used. Filtered seawater caused spawning in 100% of females and 67% of males and 50% of females and 25% of males injected with serotonin (10^{-3} M) spawned. Prostaglandin E_2 did not induce the release of gametes. Fifty percent of females injected with dopamine (10^{-3} M) spawned a small number of eggs, while the males did not respond. Males did not respond to injection of any kind of ganglia, and 20% of females spawned a few eggs in response to CG and PPG from females. Forty percent of females spawned in response to PPG from males. Injection of deionized water caused no weight changes; whereas, filtered seawater caused a reduction in weight. Prostaglandin-treated animals gained weight on the second day but lost weight over subsequent days. Only females gained weight in the dopamine treated group, but both males and females gained weight in the serotonin-treated group. With ganglionic injections, males treated with CG or PPG from males gained more weight than their female counterparts, and the females treated with CG or PPG from females gained more weight than males. The increase in mean body weight of animals was followed by a swelling and softening of the ovaries, possibly because of increased water content in the ovaries. It seems likely that uptake of water in the ovary is a physiological precursor to spawning.

KEY WORDS: *Haliotis australis*, spawning, ganglionic extracts, other neurotransmitters

INTRODUCTION

The control of spawning is essential for the efficient use of hatchery facilities and to the success of an aquaculture operation (Hahn 1989). Artificial induction of spawning allows the production of larvae over a longer period than would be possible relying solely on natural spontaneous spawning. Several methods of artificial induction of spawning in abalone have been tried over the years, some have been successful and others of limited use. Commonly used methods to induce broodstocks of abalone to spawn include immersion in ultraviolet (UV) irradiated or hydrogen peroxide (H_2O_2) treated seawater for several hours (Kikuchi & Uki 1974, Morse et al. 1977). Both of these methods seem to induce spawning by a similar mechanism; that is, generation of hydroperoxy free radicals, $HOO^{\cdot}$, or peroxy diradicals, $^{\cdot}OO^{\cdot}$, in the seawater that activate the natural enzymatic synthesis of prostaglandins in the abalone (Morse et al. 1977), although the active molecule is produced differently (Hahn 1989). Prostaglandins, as well as some amines produced by nerve cells, are considered to play an important role in spawning in other molluscs as well (Khotimchenko & Deridovich 1991).

The yellowfoot abalone, *H. australis*, is the preferred species for New Zealand abalone farmers who want to produce cocktail size (60–70 mm) abalone for export, because the color of the foot makes them more acceptable on international markets (Moss 1998). The abalone reaches sexual maturity at about 70–90 mm (Poore 1972) and can be conditioned to ripeness in the hatchery by holding at constant temperature and *ad libitum* feeding (Moss 1998, Kabir et al. 1999). With the abalone aquaculture industry expanding worldwide, the demand for regular production of high-quality larvae is increasing. Existing methods for spawning broodstock are often unreliable, species specific, and frequently result in the spawning of poor quality eggs leading to high larval mortality in cultures (Moss 1998, pers. observ.). Spawning induction of *H. australis* by the H_2O_2 method resulted in an overall spawning rate of 17% for males and 10% for females (Moss 1998), which is very poor.

The present study aimed to investigate the effectiveness of different chemicals, neurotransmitters, and ganglionic extracts on the spawning induction in *H. australis*. Another objective was to identify a suitable solvent for the preparation of solutions of chemicals or ganglionic homogenates, because there was evidence that the most commonly used solvent, seawater, itself caused spawning in other abalone species (Anon 1990). We also wanted to identify neurotransmitters, which may trigger egg maturation in this species.

MATERIALS AND METHODS

Abalone Collection and Conditioning

Yellowfoot abalone, *H. australis*, (length 84.44 ± 1.07 mm, weight 111.32 ± 4.13 gm, mean ± SE) were collected from Warrington Beach in the Blueskin Bay area of the Otago coast of New Zealand and conditioned at 15°C for 150 days (effective accumulative temperature ≥ 1,400°C-days) in the holding facilities of a commercial abalone farm at Warrington. They were fed an artificial diet, Makara (Promak Technology, New Zealand), *ad libitum* and exposed to a 12-h dark and 12-h light photoperiod. Throughout the conditioning period, gonadal development was evaluated subjectively and classified into four levels of gonad index from 0 (unripe) to 3 (gravid) (Ebert & Houk 1984). Most of the abalone reached the gravid stage after the conditioning and were spawnable.

Trial 1: Oocyte Maturation Assays

Samples of gonad, haemocoel fluid, and cerebral (CG) and pleural-pedal (PPG) ganglia from ripe, unspawned, and spawned

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males and females of *H. australis* were collected, frozen in liquid nitrogen, and stored at −80°C. Ganglia were warmed slowly and homogenized on ice in 1.0 mL of 0.2 μm filtered seawater per 10 ganglia using a glass-to-glass tissue homogeniser. Gonads were also homogenized, and the homogenates were centrifuged (8,000 rpm, 20 min), and the supernatant was used for the assay.

One fully gravid female from the conditioned stock was dissected, gonad removed, and macerated. Small pieces of gonad, together with free oocytes were placed in wells of a tissue culture dish and exposed to the different treatments (different gonad extracts, hemocoel fluids, cerebral and pleural-pedal ganglionic extracts, hydrogen peroxide, fresh seawater, and deionized water) with three replicates per treatment. Oocytes were monitored continuously using an inverted microscope for any evidence of the occurrence of germinal vesicle breakdown (GVBD), extrusion of polar bodies, and change in shape.

Trial 2: Effects of Freshwater and Seawater Injection on Induction of Spawning

Twenty-four gravid abalone (3 males and 3 females per treatment) were kept individually in 2.0-L containers with individual water flow. One group was not treated and used as a control; whereas, other groups were: (1) sham injected everyday for 3 days; (2) injected with filtered (0.2 μm) seawater; and (3) injected with deionized water. The latter two treatments were injected once per day over 3 days (1 mL/abalone) into the hemocoel posterior to the head region using a TERUMO® needle 26G × ½" (0.45 × 13 mm).

Trial 3: Effects of Serotonin, Dopamine, and Prostaglandin E_2 on Induction of Spawning

Twenty-four gravid abalone (4 males and 4 females per treatment) were kept individually in 2.0-L containers with individual water flow and monitored as described above. Serotonin (5-hydroxytryptamine), dopamine (3-hydroxytyramine), and prostaglandin E_2 (Sigma Chemicals, St. Louis, MO) at a concentration of 10^{-3} M for serotonin and dopamine, and 10^{-5} M for prostaglandin E_2 were injected once per day over 3 days (1 mL/abalone) into the hemocoel posterior to the head region. These doses were selected based on the reports in other molluscs (Martinez et al. 1996). Solutions were made using deionized water as a solvent.

Trial 4: Effects of Different Ganglionic Suspensions on Induction of Spawning

Forty fully gravid abalone (5 males and 5 females for each treatment group) were used and maintained as described above. Each treatment group was injected with: (1) cerebral ganglia from males (CGM); (2) cerebral ganglia from females (CGF); (3) pleural-pedal ganglia from males (PPGM); or (4) pleural-pedal ganglia from females (PPGF). Ganglia were collected from the fully ripe wild yellowfoot abalone and were frozen in liquid nitrogen and stored at −80°C. Before use, the ganglion were thawed and homogenized on ice with deionized water in a glass-to-glass tissue homogenizer at a concentration of one ganglia per 0.1 mL of solution. The homogenates were injected once a day over 3 days, at a dose of 0.1 mL per abalone. This dose was selected after Yahata (1973), where he diluted one ganglion per 0.1 mL and injected into the abalone.

Spawning activity was monitored continuously, and the weight of the abalone was measured at 24-h intervals. The response was recorded as positive when gametes were released. The quality of the gametes was monitored microscopically.

Handling of the Abalone

All the abalone used for the trials were subjected to gentle detachment from the tank, inversion on blotting paper to drain branchial water, weighing, and return to the respective containers. The weight for each abalone was recorded at the start and every 24 h up to the end of the experiment. The weight of the abalone was standardized considering the initial weight for the treatment group as 100%.

Statistical Analyses

The spawning results are treated as a logistic regression with the treatment and sex as factors using the regression model: proportion of spawning = exp (y) / [1+ exp (y)] + binomial error, where y = constant + treatment effect + sex effect. It assumes that the probability of spawning for individual abalone is constant. The models are found to fit the data adequately, and the results are considered significantly different if $P < 0.05$.

RESULTS

Trial 1: Oocyte Maturation Assays

The oocytes were irregular in shape when placed in the wells but became spherical within 20 min. Afterward, no change in shape, motile response, or release of polar bodies was observed for any of the treatments and replications.

Trial 2: Effects of Freshwater and Seawater Injection on Induction of Spawning

Filtered seawater caused spawning in all females and two out of three males. One female spawned in the control group. There was no spawning in other treatment groups (Fig. 1). Analysis of deviance (Table 1) indicated that treatments have a significant effect on spawning ($P < 0.05$) with the spawning by the filtered seawater differing from others. The sex had no significant effect ($P > 0.05$). Filtered seawater resulted in a loss of body weight; whereas, deionized water caused no weight change. Based on this result, deionized water was used as a solvent for further preparation of chemicals and ganglionic suspensions.

Trial 3: Effects of Serotonin, Dopamine, and Prostaglandin E_2 on Induction of Spawning

Serotonin-injected animals gained weight over time, and two females and one male spawned (Fig. 2). Two out of four females treated with dopamine spawned a small number of eggs and gained weight; whereas, no males responded and lost weight. Prostaglandin E_2 did not induce the release of gametes, and the treated abalone gained weight on the second day but lost weight over subsequent days. Analysis of deviance (Table 1) indicated that treatments have a significant effect on the spawning ($P < 0.05$) with the spawning by serotonin differing from others. The sex had no significant effect ($P > 0.05$).

Trial 4: Effects of Different Ganglionic Suspensions on Induction of Spawning

Only one out of five females spawned a few eggs in response to CGF and PPGF, and two out of five females spawned with

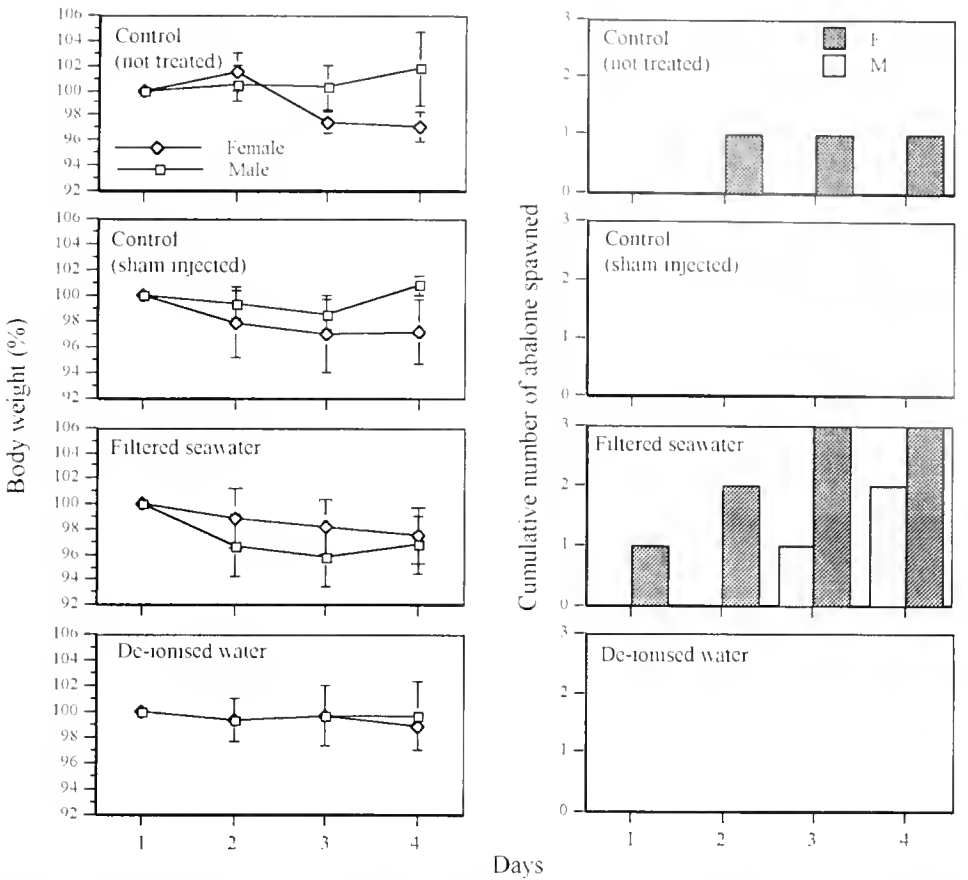

Figure 1. Changes in the mean body weight (± SE) (from a standardized initial mean weight of 100%) and cumulative spawning records of *H. australis* with different treatments.

PPGM. Abalone injected with CGM did not spawn (Fig. 3). Males treated with CGM and PPGM gained more weight than their female counterparts, and females treated with CGF and PPGF gained more weight than males. Analysis of deviance (Table 1) indicated that treatments have no significant effect on the spawning ($P > 0.05$), but the sex had significant effect ($P < 0.05$), with females differing significantly from the males.

DISCUSSION

Water temperature is considered as the main exogenous factor that regulates the reproductive cycle in many marine gastropods (Webber 1977, Hahn 1989). The process used to induce gonad ripeness and spawnability in adult abalone requires a number of exogenous and endogenous factors. The most common method to

TABLE 1.

Analysis of deviance for the spawning experiments in *H. australis* using treatments and sex as factors.

		Goodness of Fit			Chi Square Test for Term		
Trial Number	Source	Residual Deviance	DF	P	Residual Deviance	DF	P
Trial2	Constant	19.3540	7	0.0071			
	+Treatment	3.1754	4	0.5289	16.1786	3	0.0010*
	+Sex	0.0005	3	1.0000	3.1749	1	0.0748
	Constant	7.4817	5	0.1872			
	+Treatment	0.5413	3	0.9097	6.9404	2	0.0311*
Trial3	+Sex	0.0001	2	1.0000	0.5412	1	0.4619
	Constant	9.2685	7	0.2339			
	+Treatment	6.2734	4	0.1796	2.9951	3	0.3924
Trial4	+Sex	0.0003	3	1.0000	6.2731	1	0.0123*

Nonsignificant P value for the "goodness of fit" confirms the model fits adequately. "Chi square test for term" indicates the effect of treatments and sex on spawning and is considered significantly different if $P < 0.05$ (marked with asterisks).

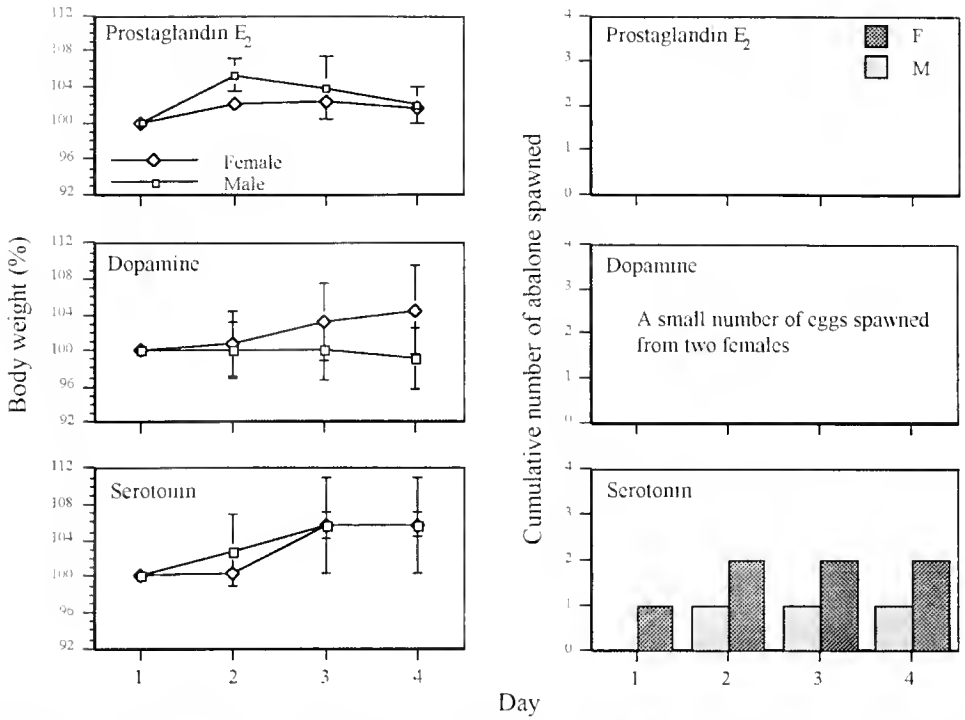

Figure 2. Changes in the mean body weight (± SE) (from a standardized initial mean weight as 100%) and cumulative spawning records of *H. australis* with different chemicals.

bring abalone into a ripe condition for breeding is to provide adults "with good living conditions (tailored to the ecology of the particular species) and *ad libitum* feeding" (Hahn 1989). The effect of temperature on abalone reproduction has been quantified in *H. discus hannai* (Uki & Kikuchi 1984) and *H. australis* (Moss 1998, Kabir et al. 1999). Our previous study (Kabir et al. 1999) showed that 21 wks of conditioning at 15°C (EAT ≥ 1,400°C-days) with *ad libitum* feeding induces gonad ripeness into a fully mature stage. Moss (1998) also reported 21 wks of conditioning at 15°C temperature was required for full gonad growth and initiation of spawning in *H. australis*, but this time may be reduced to 14 wks if the animals are held at 18°C water temperature. For the present experiment, the abalone were reared at 15°C for 21 wks following the result of Kabir et al. (1999). After conditioning, the gonad was bulging across the shell with a rounded tip at the end of a conical appendage; the ovary was brown to blue-gray, and the testis was milky white to creamy in color.

Oocytes treated with either ganglionic extract, hemocoel fluid, gonad extracts, or chemicals did not produce any motile responses or show any release of polar bodies. Saleuddin et al. (1983) reported that the freshly isolated oocytes from the ovotestis of *Helix aspersa* (Mollusca) showed amoeboid movement when treated *in vitro* with the extract of cerebral ganglia. They postulated that this form of induced motility might be associated with ovulation. On the other hand, Coggeshall (1972) suggested that ovulation in *Aplysia* is caused by the muscle contaction brought about by egg-laying hormone. Mature oocytes in the abalone do not undergo germinal vesicle breakdown until spawning is induced, and the oocytes are squeezed out of the gonad by the rapid contraction of the columnar muscle (Hahn 1990). In the present study, the inactivity of the oocytes may be attributable to the failure of oocytes to have matured sufficiently and they were, therefore, not ready for ovulation, or the isolation procedure caused invisible damage (chemical) to apparently healthy looking oocytes (Saleuddin et al. 1983). Another possibility is that the donor *H. australis* (from where the gonad, ganglia, and hemocoel fluid were collected) were not sufficiently mature and lacked the required amount of hormone to induce the oocytes. The exact mechanism of ovulation in abalone requires further investigation.

A preliminary study showed three out of five blackfoot abalone, *Haliotis iris*, spawned when a filtered seawater injection was used as a control. This prompted us to investigate the use of seawater as a control or its use as a solvent to homogenize different ganglia or other chemical solutions for the spawning experiment of *H. australis*. In the present experiment, injection of filtered seawater into the hemocoel caused spawning that is significantly different from other treatment groups ($P < 0.05$). Injection of 3.7% calcium chloride, sterilized or filtered seawater into the head and foot of *H. discus hannai* has also been observed to cause spawning (Anon. 1990). Yahata (1973) did not observe any spawning when 0.6% NaCl solution was injected into *Nordotis discus*, but it resulted in weight loss in the abalone. Thus, it seems that seawater or other salt solutions often induce spawning in *H. australis* and other abalone perhaps by stimulating the nervous systems. Therefore, we must be cautious in selecting a solvent to homogenize ganglia or to prepare other chemical solutions for spawning experiments with abalone.

The use of neurotransmitters and other chemicals in induction of spawning in molluscs (Vélez et al. 1990, Ram et al. 1993, Martinez et al. 1996) and of abalone (Morse et al. 1977) has been reported. Serotonin has been shown to be one of the most effective spawning inducers in some molluscs (surf clam Hirai et al. 1988, scallop Matsotani & Nomura 1982, China clam Alcazar et al. 1987, and giant clam Braley 1985). Therefore, the release of serotonin may play a role in abalone spawning. Prostaglandins (PG) have been suggested to be involved in the release of gametes

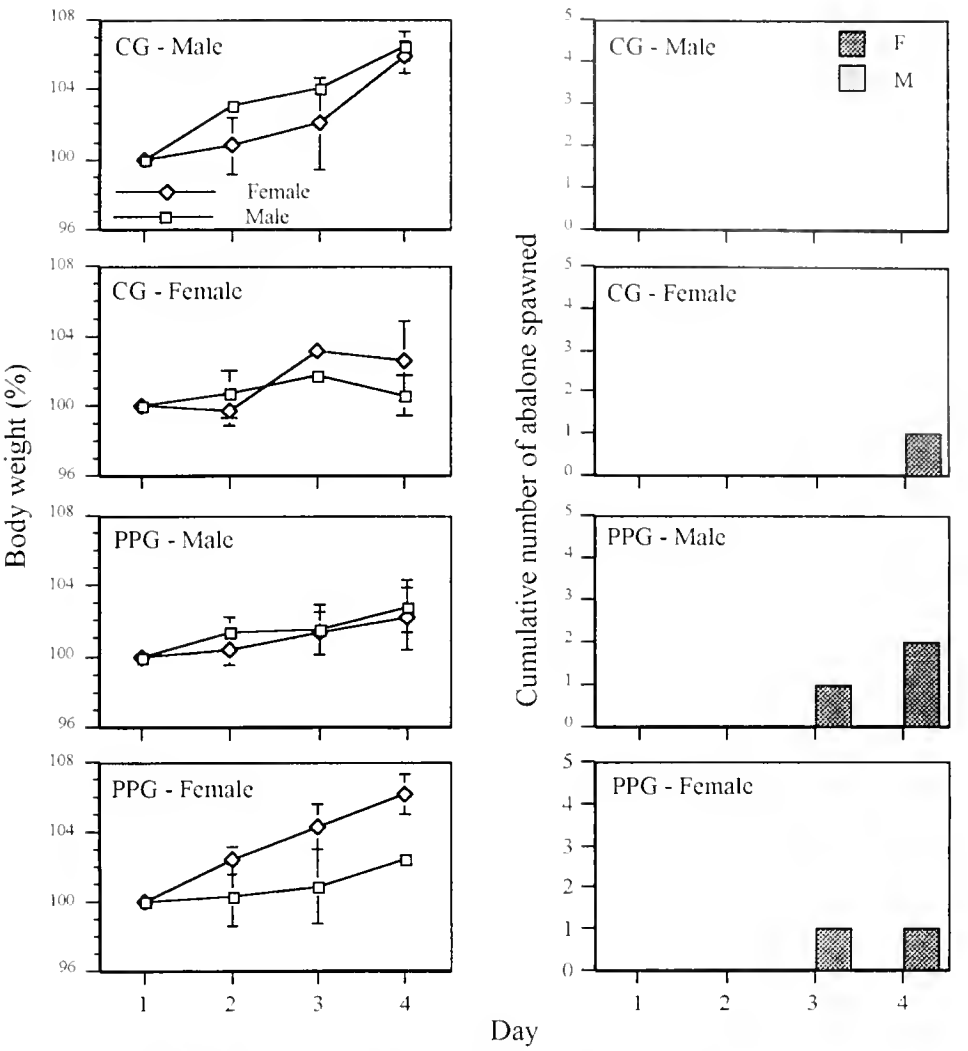

Figure 3. Changes in the mean body weight (± SE) (from a standardized initial mean weight of 100%) and cumulative spawning records of *H. australis* with different ganglionic suspensions.

(Morse et al. 1977). They showed that the ultraviolet (UV) irradiation of seawater or addition of hydrogen peroxide (activator of prostaglandin synthesis) to seawater induced spawning in male and female red abalone, *H. rufescens*. They were also able to induce spawning by adding the vertebrate hormone prostaglandin E or F (3×10^{-12} M) to the water. These hormones induced spawning in 38 to 47% of gravid abalone. Martinez et al. (1996) used 2×10^{-6} to 2×10^{-8} M prostaglandin E_2 for the spawning of scallop, *Argopecten purpuratus*. Kunigelis and Saleuddin (1986) reported that introduction of nanogram quantities of PGE_2 into the female genital opening of mated *Helisoma* increased the number of egg masses and the number of eggs per mass. The intrahemocoelic administration of thousand-fold higher concentration of PGE_2 failed to induce spawning. They postulated that the action of prostaglandin is localized and hormonally mediated. The failure of spawning by PGE_2 injection in this experiment supports the findings of Kunigelis and Saleuddin (1986), but a dose response experiment with different administration protocol in *Haliotis* sp. is essential to evaluate its role in abalone spawning.

In the present experiment to test the effect of ganglionic extracts, the treatments had no significant effect on spawning ($P >$ 0.05) Injections of crude homogenates of the cerebral ganglia into ripe females did not induce full spawning in ripe *H. discus hannai* (Yahata 1973). In their experiment, two out of five abalone injected with CG homogenates spawned a small amount of clustered eggs; however, there was a considerable gain in the mean body weight from an increase in water uptake without any noticeable change in the ovary. Hahn (1989) postulated that A-cells from cerebral ganglia of probably regulate vitellogenesis in females, but this ganglion does not contain a factor that is involved in the induction of spawning. Yahata (1973) also found that the injection of pleural-pedal and visceral ganglia crude homogenate into female *H. discus hannai* caused a large swelling and softening of the ovary and spawnings after two injections. Therefore, these ganglia may produce and release factors that induce spawning in abalone. Histological examination of the PPG showed several cell types producing abundant neurosecretory material that seemed to vary in relation to the reproductive cycle (Hahn 1990). The handling stresses (such as detaching, injection, and weighing) of the abalone on spawning were not evaluated in the present experiment. A recent study showed that handling for weighing may produce 7 to 17% weight reduction in adults of *H. iris* (Ragg et al. 2000). An

increase in body weight was observed in the present experiment with ganglionic suspensions and other neurotransmitters. These increases in the body weight may be caused by the increased uptake of water in the ovary, which indicates that the uptake of water by the gonad may be essential to bring about spawning (Yahata 1973).

The weak or almost no response of the ganglionic extracts in the spawning of *H. australis* in the present experiment raises a number of issues. First, the maturity of the animals from where the ganglia were collected must be examined. The abalone were collected from Warrington Beach, and those with bulging gonad above or near the shell edge were taken. Abalone with such bulging gonads are considered to be fully ripe (Ebert & Houk 1984), but this does not provide information on the oocyte maturation stage. It could be possible that in the wild population, abalone with fully developed gonads may not necessarily be spawnable and lack factors in the ganglia that are responsible for spawning. Second, the abalone that were injected came from the conditioned stock (1,400 C-days). It has been shown histologically that after such conditioning period oocytes become fully grown, and the abalone are spawnable (Kabir et al. 1999). Thus, the abalone used in this experiment were well gravid. Third, the dose of the ganglia (i.e., 1 ganglion per 0.1 mL = 1 injection) was selected following the protocol of Yahata (1973). This dose may not be effective for this species. Therefore, it requires further investigation with the ganglia to be collected from conditioned individuals and employed with different doses and combinations.

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REPRODUCTIVE PERFORMANCE INDICES BASED ON PHYSICAL CHARACTERISTICS OF FEMALE BLACKLIP ABALONE *HALIOTIS RUBRA* L.

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ABSTRACT Selection of abalone broodstock from the wild is often based on external appearances. The criteria used are size, color, and shape of the gonad. However, animals selected on external appearance are known to vary in egg fertilizability, hatching rates, and larval survival. The present study was instigated to develop easily useable indices, based on physical characteristics, for assessing the potential reproductive performance of female abalone. Analysis was conducted on wild-caught, artificially propagated blacklip abalone *Haliotis rubra* L., obtained from Australian coastal waters (142°15′; 38°21′S). Females were spawned using a combination of the desiccation and the UV irradiated methods and shell characteristics; length (SL), width (SW), height (SH), and total weight (W) of the spawners were determined. The fertilized eggs from each spawn were incubated, hatched, and larvae were reared separately in a flow-through system. The criteria used for assessing reproductive performance were fecundity, fertilizability, hatchability, and pre-settlement survival. The results show that fertilizability was positively correlated to hatchability and larval survival ($P < 0.01$). Furthermore, shell physical characteristics and weight can be used as predictors of reproductive performance. Accordingly, a number of polynomial regressions incorporating SL, SW, SH, W, and egg characteristics to fertilizability, and SL and W to fecundity, were developed.

KEY WORDS: blacklip abalone, *Haliotis rubra*, broodstock assessment, shell characteristics, reproductive performance

INTRODUCTION

Abalone (*Haliotis* spp.) is commercially exploited for its valuable meat and shell. Long-term exploitation of this group has resulted in the depletion of wild stocks in many areas of the world. To fulfill the high demand and because abalone take a long time to reach marketable size in the wild, culture of abalone has become a viable alternative to fishing wild stocks.

Australia is one of the main exporters of wild-caught abalone, contributing about 60% to world production. It is now in a prime position to become a world leader in the production and export of cultured abalone (Flemming & Hone 1999).

In abalone culture, wild-caught broodstock are used extensively. Before this study, the suitability and selection of broodstock from such wildstock has been based on the external appearances of mature animals. The main criteria used in such selection are size, color, and shape of the gonad. However, animals selected based on these criteria are known to vary widely in reproductive performance.

The present study attempts to develop suitable indices for broodstock assessment based on the physical characteristics of female blacklip abalone. It is expected that this will provide a method for performance estimation and will contribute to knowledge on broodstock selection in abalone aquaculture. Accordingly, this paper is based on the results of the spawning of 8 and 11 female blacklip abalone, *Haliotis rubra* L. in the spawning season of 1997 to 1998 and 1998 to 1999, respectively. Furthermore, in the 1999 to 2000 spawning season, the results of five females spawned were used for validation of the models developed based on observations in the previous spawning seasons.

MATERIALS AND METHODS

Spawning

Broodstock were obtained from coastal waters (142°15′E; 38°21′S) during the spawning seasons of 1997 to 1999 and 1998 to 1999. Spawning was conducted during the routine spawning operations of Southern Ocean Mariculture, Port Fairy, Victoria, Australia. To reduce stress, except for total weight, all physical characteristics of females used in the present studies were recorded after spawning. Spawning was triggered by using a combination of desiccation and the UV irradiated methods (Kikuchi & Uki 1974). Spawning was conducted using a flow-through system (velocity of 100 mL min^{-1}). In this study, to eliminate the influence of males, sperm from a number of males were pooled together, and females were spawned individually.

Spawned eggs and sperm were immediately separated from the spawners with a siphon, washed with 1μ of UV-treated seawater of 35‰ salinity, and the spawned eggs were concentrated into a known volume of clean seawater. The water containing the eggs was gently stirred to ensure a homogenous distribution of eggs through water column, and three subsamples were taken for estimating fecundity. Artificial fertilization was effected by mixing known numbers of newly released eggs with appropriate numbers of fresh sperm at an approximate ratio of 5 sperm/egg. Incubated eggs and larvae from each female were kept separately. The hatching stage was run using a static system, and hatched larvae were transferred into rearing containers soon after hatching. Larval rearing was done using a flow-through system (velocity of 80 mL min^{-1}), for 5 days with the extent of presettled larvae being determined by formation of the third tubule on the cephalic tentacle (Hahn 1989). Hatchery procedures followed that described elsewhere (Hone et al. 1997).

Parameters

Such parameters as physical characteristics of adults, number of eggs (fecundity), diameter of eggs, fertilizability, hatchability, and presettlement survival were recorded for 19 female blacklip abalone. Physical features including shell length (SL), shell width (SW), shell height (SH), and total weight (W) of each female spawner were recorded. Shell measurements were made with a Vernier caliper (to the nearest 0.05 mm). Conventional measurements of the shell used in this study are shown in Figure 1. Weight was recorded using a scale (to the nearest 0.01 g), and egg diameter was measured using an eyepiece graticule with a compound

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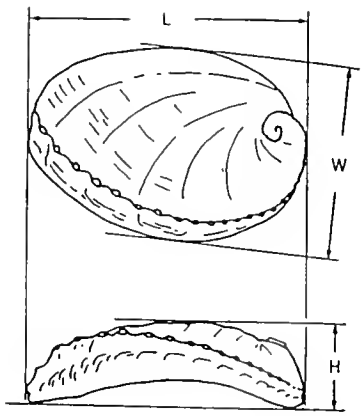

Figure 1. Conventional shell measurement of length (L), width (W), and height (H). (Mgaya & Mercer 1994).

microscope (to the nearest 0.5 μ). At least 150 eggs from each spawned female were measured, and means for individual females were used in regression analysis. Fertilizability was estimated by determining the number of fertilized eggs in a subsample of 1 mL taken after mixing eggs and sperm. For each spawner, five such subsamples were used, and the mean was determined. Similarly, hatchability and larval survival were estimated.

Fecundity is the total number of eggs produced by an individual female.

$$\text{Fertilizability (\%)} = \frac{\text{Total number of fertilized eggs}}{\text{Total number of eggs produced}} \times 100$$

$$\text{Hatchability (\%)} = \frac{\text{Total number of hatched larvae}}{\text{Total number of fertilized eggs}} \times 100$$

$$\text{Larval survival (\%)} = \frac{\text{Total number of survived larvae}}{\text{Total number of hatched larvae}} \times 100$$

Statistical Analysis

Scatter plots were used to observe the distribution of the data. Statistical relationships between physical characteristics and each of the reproductive performance indicators were explored using the software package Minitab version 12.1. The relationships then tested for, between parameters, were linear, curvilinear and the second- and third-order polynomial.

TABLE 1.

Physical characteristics of female abalone spawned (19) and the mean (±SE) of fecundity, fertilizability, hatchability, and larval survival from the spawning seasons 1997/98 and 1998/99.

Parameters	Mean (SE)	Range
Shell length (mm)	128.97 (1.70)	120.05–141.50
Shell width (mm)	103.64 (1.80)	90.95–121.00
Shell height (mm)	40.35 (1.52)	29.65–52.35
Total weight (g)	383.81 (19.51)	274.37–598.52
Fecundity (10^6)	3.51 (0.43)	0.54–5.92
Fertilizability (%)	91.36 (1.73)	70.47–97.30
Hatchability (%)	87.54 (1.21)	75.73–96.87
Larval survival (%)	79.41 (1.69)	66.00–88.41

RESULTS

Female Physical Features and Reproductive Performance

The legal size limit for wild-caught Victorian blacklip abalone is 120 mm. The mean (± SE), the range of the different physical features, and reproductive performance criteria of the 19 female abalone used in the study are given in Table 1.

Relationships of Shell Characteristics to Reproductive Criteria

The potential statistical relationship of the shell characteristics to the female reproductive criteria; namely, fecundity, fertilizability, hatchability, and larval survival, were explored. The resulting relationship, in the form of a correlation matrix, is given in Table 2. It was evident that only shell width and total weight were positively correlated to hatchability. None of the other shell characteristics, when individually considered, were correlated to any of the reproductive criteria in the study. Similarly, multiple regression analysis incorporating shell characteristics did not result in a statistically significant relationship to performance predictors.

It is also clear from Table 2 that fertilizability was positively correlated to hatchability ($r = 0.64$, $P < 0.01$) and to larval survival ($r = 0.65$, $P < 0.01$). Also, hatchability was positively and linearly correlated to larval survival ($r = 0.55$, $P < 0.05$) (Table 2).

TABLE 2.

Correlation matrix of relationships of female blacklip abalone physical characteristics to the different indices of reproductive performances ($N = 19$).

	SL	SW	SH	W	Fc	Ft	Ht	Sv
SL	1.00	0.82**	0.50*	0.81**	0.06 NS	0.08 NS	0.44 NS	0.17 NS
SW		1.00	0.54*	0.91**	0.06 NS	0.25 NS	0.52*	0.39 NS
SH			1.00	0.47*	0.26 NS	−0.28 NS	0.02 NS	0.09 NS
W				1.00	0.04 NS	0.14 NS	0.46*	0.26 NS
Fc					1.00	−0.15 NS	−0.28 NS	−0.03 NS
Ft						1.00	0.64**	0.65**
Ht							1.00	0.55*
Sv								1.00

Significance level is indicated as $P < 0.01$ (**), $P < 0.05$ (*) or not significant (NS).
Abbreviations: SL = shell length; Ft = fertilizability; W = total weight; SW = shell width; Ht = hatchability; SH = shell height; Sv = larval survival; Fc = fecundity.

TABLE 3.

Correlation matrix, partial coefficients of the combinations of physical characteristics to the indices of reproductive performance of female blacklip abalone (n = 19).

	Fecundity	Fertilizability	Hatchability	Larval Survival
SL/SW	0.82***	0.83***	0.77***	0.83***
SL/SH	0.50*	0.55*	0.55*	0.50*
SL/W	0.81***	0.81***	0.76***	0.81***
SW/SH	0.54*	0.65**	0.62**	0.55*
SW/W	0.91***	0.91***	0.88***	0.90***
SH/W	0.47*	0.53*	0.51*	0.46*

Significance level is indicated as $P < 0.001$ (***) $P < 0.01$ (**), $P < 0.05$ (*). For legends, see Table 2.

Hatchability (Ht) can be predicted by using performance indicator fertilizability (Ft), and its relationship is best described by the equation, Ht = 35.1 + 0.575 Ft (R^2 = 0.89, $P < 0.001$). The relationship between fertilizability (Ft) and larval survival (Sv) is described by the equation Sv = 7.4 + 0.802 Ft (R^2 = 0.73, $P < 0.001$). Furthermore, hatchability (Ht) can also be applied to estimate survival (Sv), and their relationship is described by the equation, Sv = −0.272 + 1.22 Ht (R^2 = 0.79, $P < 0.001$).

The regression relationships between different combinations of shell characteristics and reproductive performance indicators are given in Table 3. Partial correlation coefficients indicated that combination of SL/SW and SW/W are strongly correlated to fecundity, fertilizability, hatchability, and larval survival (in all cases $r > 0.77$, $P < 0.001$). A combination of SW/SH was also found to be significantly correlated to fertilizability ($r = 0.65$, $P < 0.01$) and to hatching rate ($r = 0.62$, $P < 0.01$), but was less significantly correlated to fecundity ($r = 0.54$, $P < 0.05$) and to larval survival ($r = 0.55$, $P < 0.05$).

However, none of the above relationships were linear. Shell characteristic; length, height, and width bear a quadratic relationship to fertilizability (Table 4). On the other hand, relationships between weight to fertilizability are best described by a cubic function. The quantitative relationships between female physical characteristics (SL, SH, SW, W) and fertilizability, and the relationship between SL and W and fecundity are shown in Table 4.

The quantitative relationships between shell length and total weight to fecundity are illustrated in Figure 2. The fecundity (Fc) of blacklip abalone was also related quadratically to length (SL) and total weight (W). Their relationships are best described by the following equations.

$$Fc = -622.238 + 9.492\ SL - 0.0359\ SL^2 (R^2 = 0.59, P < 0.01)$$

$$Fc = -26.628 + 0.147\ W - 1.67E\text{-}04\ W^2 (R^2 = 0.66, P < 0.01)$$

Egg Quality and Reproductive Performances

Egg quality in terms of yolk diameter and the ratio between yolk and total diameter could also be used as a predictor of fertilization (Figs. 3, 4). The relationship between yolk diameter (YD) and fertilizablity (Ft) is best described by the equation Ft = −9028.77 + 97.557 YD− 0.261YD^2 (R^2 = 0.92, $P < 0.001$). In addition, fertilizability (Ft) was correlated to the ratio of yolk diameter and total egg diameter (TED), the relationship being Ft = − 2862.67 + 6786.67 TED–3891.62 TED^2 (R^2 = 0.77, $P < 0.001$).

Validation of Indices

Validation of the present indices was done using the data from the 1999 to 2000 spawning seasons. The results of these particular spawnings, predicted and observed parameters are given in Table 5. The mean predicted fertilizability for the five females differed from the observed value by 5.7%. In all instances except in one female (−1.8%) the predicted fertilizability was higher (6.7 ± 2.2%). The predicted fecundity, however, was more variable, particularly with respect to the estimation based on one female (Table 5).

DISCUSSION

In abalone culture, selection of broodstock is mainly based on gonad appearance. The mantle and foot are manipulated to expose the gonad, which is markedly swollen, blunt and rounded at the tip indicating gravidity (Grant 1989). Abalone are judged to be ready for induced spawning by the amount of swelling of the gonad. Without a careful handling procedure, broodstock selection based on this technique is bound to stress the animals. Therefore, a quick method of assessment with minimal disturbance is desirable. Present results demonstrate that female physical characteristics can be used for predicting the spawning performance.

Regression analysis is one of the most widely used statistical

TABLE 4.

Results of regression analysis of shell and egg characteristics and total weight to fertilizability and fecundity (1st order equation, $Y = a + bX$; 2nd order equation, $Y = a + b_1X + b_2X^2$ and 3rd order equation, $Y = a + b_1X + b_2X^2 = b_3X^3$).

Independent Variable (X)	Dependent Variable (Y)	a	b_1	b_2	b_3	n	R^2	P
SL	Ft	−1344.890	−23.515	−0.096	—	12	0.86	0.000
SW	Ft	−355.011	7.858	−0.034	—	11	0.66	0.013
SH	Ft	36.609	3.482	−0.051	—	12	0.87	0.000
W	Ft	923.485	−7.026	0.019	−1.8E-05	14	0.81	0.000
YD	Ft	−9028.770	97.557	−0.261	—	11	0.92	0.000
TED	Ft	−5495.800	52.797	−0.124	—	12	0.71	0.003
Ratio YD/TED	Ft	−2862.67	6786.67	−3891.62	—	13	0.77	0.000
SL	Fc	−622.238	9.492	−0.035	—	15	0.59	0.004
W	Fc	−26.628	0.147	−1.70E−04	—	14	0.66	0.002

YD = yolk diameter (μm); TED = total egg diameter; Other legend, see Table 2.

tools, because it provides a simple method for establishing a functional relationship between variables (Chatterjee & Price 1977). Linear relationship has been commonly used in performance studies on other species (Shepherd et al. 1992, Estay et al. 1999). In the present study, a polynomial regression model successfully estimated the reproductive performance of female blacklip abalone. As indicated here, most of the relationships are best fitted with nonlinear models.

Correlation analyses showed that some combinations of shell features correlated well with performance (Table 3). As indicated by correlation coefficients, we would expect bigger animals and ones with a flattened shell to be a good indication for performance estimation. The same trend was also observed in bigger and heavier animals. This conforms with other observations that larvae from blacklip abalone broodstock having bigger and wider sizes usually show a faster growth rate (SOM, pers. comm.).

Fecundity is one important performance criterion. The relationship of fecundity to size (length or weight) has important consequences for both fisheries management and aquaculture. Determinations of the size limit, which allows the harvest of immature animals, in abalone fisheries, will affect egg production. In aquaculture, however, fecundity plays a role in routine hatchery operations. Estimations of egg production could allow hatchery operations to be optimized.

In blacklip abalone populations, fecundity can be approximated by nonlinear relationships with length and has been described by a power function ($Y = aX^b$) by others (McShane 1990, Nash et al. 1994). Exponential increase of fecundity with increasing length has also been reported for other abalone species, *Haliotis roei* (Wells & Kessing 1989), *H. midae* (Newan 1967), *H. tuberculata* (Hayashi 1980), and *H. laevigata* (Wells & Mulvay 1995). In addition, McShane (1990) and Nash et al. (1994) suggested that fecundity is size dependent rather than age dependent. In a separate study, on the other hand, Prince et al.(1988) argued that fecundity of *H. rubra* was closely related to age rather than length. The present study indicates that fecundity varied widely, even among a small range of sizes (Table 1), and its relationship to length and weight was best described by a second-order polynomial function. As shown by the quadratic model (Fig. 2), fecundity increases with increasing size, but, decreased as animals grew beyond a certain size. This change may be brought about by a limited release of eggs by older and bigger animals, or simply because the number of eggs produced decreases as the animals grow beyond a certain size. It would be interesting to investigate whether or not there is a tendency for bigger animals to spawn early (or late) in the season and *vice versa*; a trend that may be of use in improving hatchery production.

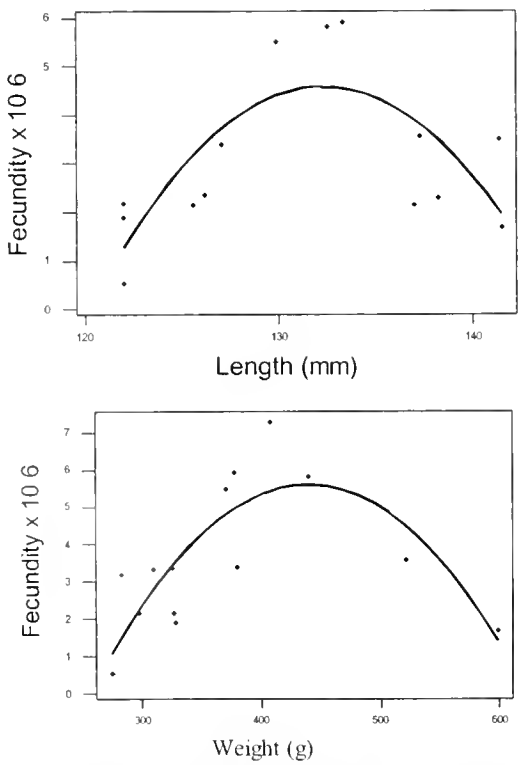

Figure 2. Regression plot of shell length versus fecundity (A) (R^2 = 0.59, P <0.01) and weight versus fecundity (B) (R^2 = 0.66, P < 0.01)

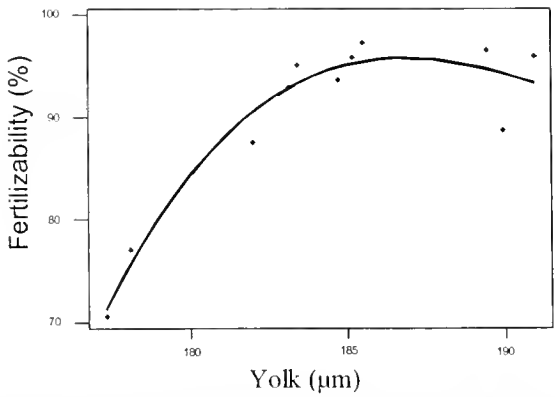

Figure 3. Quadratic model of mean yolk diameter (X) versus fertilizability (Y) (R^2 = 0.92, P < 0.001).

The egg yolk diameter and the ratio between yolk and total diameter seemed to be another method of predicting egg fertilizability (Figs. 3, 4). For such nonfeeding larvae, as those of abalone, future spawn success is determined by egg quality (size and biochemical content). It has been widely known that egg biochemical content is size-dependent. Consequently, it is obvious that egg diameter is an important key in determining performance (Brooks et al. 1997). Investigations on other invertebrates have shown that small differences in egg size and content might have important biological consequences (George 1999). This author found that larval seastars from small eggs tend to grow and develop more

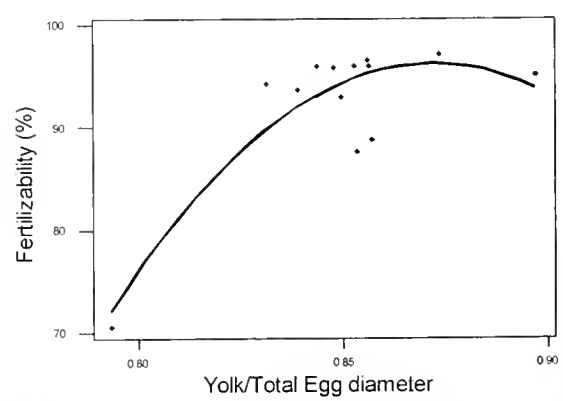

Figure 4. Quadratic model of ratio of yolk and total egg-diameter (X) versus fertilizablity (Y) (R^2 = 0.77, P < 0.001).

TABLE 5.

Shell characteristics and corresponding predicted values for fertilizability and fecundity, based on the equations in Table 4 and a comparison of the latter to those observed for five females spawned in the 1999/2000 season. For each female, the measurements given in order are shell length, shell width, shell height, and weight. Fecundity predictions are made using only length and weight.

		Predicted		Observed	
Shell Characteristics (SL;SW;SH) (mm)	**Weight (g)**	**Fecundity (10^6)**	**Fertilizability (%)**	**Fecundity (10^6)**	**Fertilizability (%)**
121.65; 105.95; 39	275	0.89; 0.94	95.0, 93.6; 94.5	0.65	92.3
127.7; 105.55; 34.8	285.95	4.13; 1.53	92.8; 93.4; 95.8	4.08	92.3
127; 94.65; 32.7	NA	NA	93.7; 82.3; 95.7	NA	91.7
130.95; 98.55; 33	360.49	4.78; 4.27	88.2; 87.2; 95.8	4.75	86.1
136;37; 101; 34.35	NA	NA	76.7; 89.7; 95.8	NA	74.0

NA = not available.

slowly than those from large eggs. According to the present findings, bigger egg size did not necessarily result in a better performance, however. A similar pattern was also noticed when the ratio of yolk diameter and total diameter was used as a fertilizability predictor. Yolk size of 182 to 188 μ and a ratio of yolk and total egg diameter between 0.83–0.87, respectively, seem to indicate the range where blacklip abalone performs best (Figs. 3, 4). It seems that a higher fertilization success is restricted to a certain range of egg sizes. A change in fertilizability beyond these limits may be attributable to age, overripe eggs or egg biochemical status.

In summary, female physical characteristics can be used to predict reproductive performance. The present results provide several easily useable indices for assessing the spawning performance of blacklip abalone. It would be useful in future to apply the present indices to a larger number of broodstock and also compare them to broodstock indices from other abalone species. From a nutrition point of view, it would be valuable to examine the relationship between egg size and biochemical content and its implications for the early life cycle of abalone.

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EFFECTS OF COMBINED EXPOSURE TO ELEVATED AMMONIA AND LOW DISSOLVED OXYGEN LEVELS IN GREENLIP (*HALIOTIS LAEVIGATA* DONOVAN) AND BLACKLIP (*H. RUBRA* LEACH) ABALONE. I. GROWTH AND MORTALITY DATA FROM SIMULATED SYSTEMS FAILURE

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ABSTRACT A growth trial was conducted on two Australian species of commercially cultured abalone to determine the effect of episodic exposure to suboptimal water quality on growth and mortality. This was primarily designed to simulate systems failure, resulting in partial or complete interruption in the normal water exchange and corresponding changes in dissolved oxygen and ammonia levels. Experimental abalone were nominally exposed, at intervals ranging from once in 6 wk to once a week, to 60% dissolved oxygen saturation and 150 μg L^{-1} un-ionized ammonia for 8 h. No significant effect on growth or mortality was found for either species, with observation of abalone behavior and food consumption indicating the exposures imposed a mild and transient stress. A more severe challenge exposure (nominally 30% dissolved oxygen and 600 μg L^{-1} un-ionzed ammonia for 8 h) at the end of the growth trial had a greater impact on food consumption and activity, but this disappeared within 24 h and did not cause any mortality after 2 days.

KEY WORDS: blacklip abalone, greenlip abalone, *Haliotis laevigata*, *Haliotis rubra*, dissolved oxygen, ammonia, pulse exposure, systems failure

INTRODUCTION

Although it is the desire of any aquaculturist to maintain a constant supply of air and water for the species under culture, forces outside human control will inevitably interrupt the supply of water during some part of the production cycle. Once the regular supply of air and water has been reduced or interrupted, a rapid decline in water quality can be expected as oxygen consumption continues and metabolites accumulate. In marine aquatic systems, this rapidly becomes problematic, because potentially toxic ammonia is the most common nitrogenous metabolite excreted by both vertebrate and invertebrate species (Colt & Armstrong 1981, Russo & Thurston 1991, Wajsbrot et al. 1991). In aqueous solution, ammonia exists as a combination of ionized and un-ionized forms, with the equilibrium between the two being governed by pH, temperature, and the ionic strength of the solution (Colt & Armstrong 1981, Meade 1985, Russo & Thurston 1991). Although un-ionized ammonia (UIA) has previously been accepted as the predominantly toxic form, there is some evidence that ionized ammonia may also exert a toxic effect (Thurston & Russo 1981, Meade 1985, Russo & Thurston 1991).

Gastropod mollusks, such as abalone, are biochemically suited to surviving periods of hypoxia or even anoxia, by using alternative biochemical pathways that are not dependent on oxygen for the generation of ATP (Brix et al 1979, Gäde & Ellington 1983, Storey & Storey 1990). This is presumably an adaptation to episodes of environmental hypoxia. It is not clear whether mollusks are similarly suited to episodes of elevated ammonia or low oxygen tensions in combination with elevated ammonia levels. Results from individual bioassays have shown that abalone are sensitive to chronic exposure to elevated ammonia or to low dissolved oxygen levels, with 5 and 50% reductions in growth rates reported at 41 and 158 μg L^{-1} UIA or 7.36 and 5.91 mg L^{-1} dissolved oxygen (DO) respectively (Harris et al. 1998, 1999a).

It is important for aquaculturists to have some knowledge of the sensitivity of the cultured species to changes in water quality that are likely to occur when the supply of water is interrupted for an extended period. Although there is an increasing body of general literature on abalone, there is a paucity of information on acute or chronic exposure to combinations of altered water quality variables. As well as the possibility of direct mortality, potential stress-related complications include secondary infections and growth reductions. Repeated episodes are likely either to augment such effects, or enable acclimation such that the abalone are better able to deal with more severe subsequent exposures.

The present trial investigated the effect on growth and mortality of episodic exposure to low dissolved oxygen levels combined with elevated concentrations of UIA in two species of abalone (blacklip, *Haliotis rubra* and greenlip, *H. laevigata*) cultured in Australia. The effect of exposure history on the response to a subsequent more severe challenge was also assessed.

METHODS AND MATERIALS

The trial was conducted at a research facility at Bicheno, Tasmania, Australia (E148°18′, S41°53′). The experimental tanks were circular fiberglass units (diameter = 70 cm, volume = 55 L). The 30 tanks used in the trial were enclosed within an area covered with shade cloth to prevent the development of algal biofilms. Each tank was individually supplied, on a flow-through basis, with sand-filtered oceanic seawater, drawn from subsurface intakes on an exposed coastline. The salinity was 34–35 g L^{-1} throughout the trial, and was not affected at any stage by freshwater runoff. Aeration was supplied through two 50-mm airstones per tank.

Experimental Abalone

The experimental abalone were obtained from commercial stock at two abalone farms in Tasmania, Australia. Blacklip abalone, 18–24 mo old (mean length = 42.9 ± 3.6 mm, mean weight = 13.00 ± 4.45 g, ± SD, N = 600), were obtained from Swansea,

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Tasmania. Greenlip abalone, 24–30 mo old (mean length = 45.8 ± 3.9 mm, mean weight = 12.21 ± 3.01 g, ± SD, N = 600), were obtained from Bicheno, Tasmania. Both species had been held in 270-L holding tanks for 3–8 wk before being randomly allocated to individual tanks after being weighed (to 0.01 g) and measured for length (to 0.1 mm, using Vernier calipers). Polyethylene tags (Hallprint, Adelaide, Australia) had previously been applied, with any missing tags replaced using a cyanoacrylate adhesive gel. Abalone were stocked at 40 animals per tank with triplicate tanks for each species per treatment. The treatments were stocked at intervals of 6–7 days, with the abalone being carefully removed by spatula, without anesthesia, from the holding tanks.

The diet used before and during the trial was based on a commercial formulation (ABCHOW), with the addition of 10% algal meal. The tanks were routinely cleaned every 4–5 days by siphoning the bulk of the organic wastes, spinning the water gently, and then siphoning off the remaining wastes that collected in the center of the tank. The abalone were not exposed to air during cleaning. Fresh food was supplied every 2 days, with the weighed ration for each tank adjusted on an *ad libitum* basis.

Growth Trial

The growth trial ran for 47 days, with ambient water quality shown in Table 1. At the intervals indicated in Figure 1, the tanks were nominally exposed to 60% of the ambient dissolved oxygen concentration and 150 μg L^{-1} UIA. The actual conditions during these exposures are given in Table 1. The first exposure for Treatment 5 was 4 h; all subsequent exposures were 8 h (starting between 8:30 and 11 AM).

Based on lengths and weights for individual animals, the growth indices given in Figure 2 were calculated as follows:

Specific Growth Rate for Length (SGR-L) = {[ln(L2) − ln(L1)]*100}/T

Specific Growth Rate for Weight (SGR-W) = {[ln(W2) − ln(W1)] × 100}/T

Microns per Day = {(L2 − L1)/1000} × T

where L1 is length at T1 (mm), L2 is length at T2, W1 is weight at T1 (g), W2 is weight at T2, and T is time in days.

Pulse Exposures

On the day of exposure, water quality (pH, temperature, DO) were recorded, a sample was collected for subsequent ammonia analysis, and then the bulk of organic wastes (80–90%) was siphoned from the tanks. When the normal water level had been restored, conditions were altered by diverting influent water, infusing industrial grade N_2 into the tanks and adding 2.5 L of ammonia stock solution (0.33 g L^{-1} of technical grade ammonia chloride in sand-filtered seawater). Preliminary trials showed that this would achieve the desired UIA concentration. All tanks were adjusted to the same volume at the start of the trial.

Submersible aquarium pumps were placed in each tank immediately before altering the water quality to ensure that the conditions were uniform throughout the water column during the pulse exposure. Initially, large volumes of N_2 were infused to reduce the oxygen level rapidly, with 60% saturation being achieved within 3 min. This concentration was subsequently maintained using small amounts of N_2 and air, infused through separate 50-mm airstones.

The return to ambient conditions was achieved by restoring influent water flow and removing the N_2. The pumps were left running in the tank for 1 h postexposure to expedite the return to ambient conditions. Time-course sampling at the start of the trial showed that DO returned to ambient levels within 1 h, and ammonia within 6 h (data not shown). The pumps were placed in control tanks, and tanks not being exposed on a given week, as for the pulsed tanks, to reduce the possibility of any confounding influence on growth.

TABLE 1.

Water quality during ambient, pulse, and challenge periods (mean ± SE). All values are based on means from replicate tanks (N = 15 for ambient, N = 12 for pulse and challenge).

Water quality variable	Sample				
		Pulse		Challenge	
	Ambient	T = 15 min	T = 8 h	T = 15 min	T = 8 h
pH					
Greenlips	8.11 ± 0.00		7.69 ± 0.02		7.73 ± 0.035
Blacklips	8.11 ± 0.00		7.79 ± 0.03		7.85 ± 0.02
Dissolved Oxygen (mgL^{-1})					
Greenlips	7.54 ± 0.01		4.34 ± 0.04		2.36 ± 0.10
Blacklips	7.54 ± 0.01		4.69 ± 0.16		2.35 ± 0.07
Temperature (°C)					
Greenlips	17.29 ± 0.03		20.61 ± 0.54		19.62 ± 0.77
Blacklips	17.23 ± 0.03		19.86 ± 0.35		19.61 ± 0.77
TAN (mg L^{-1})					
Greenlips	0.03 ± 0.01	5.02 ± 0.21	4.51 ± 0.23	19.14 ± 4.24	15.53 ± 3.60
Blacklips	0.05 ± 0.01	5.11 ± 0.22	4.69 ± 0.30	16.50 ± 2.81	16.30 ± 3.20
UIA (μg L^{-1})					
Greenlips	0.92 ± 0.23	157.31 ± 8.68	73.68 ± 6.73	625.77 ± 144.10	384.47 ± 115.23
Blacklips	1.91 ± 0.27	163.57 ± 8.98	91.73 ± 8.05	547.87 ± 95.86	372.43 ± 107.98

Because of lack of difference from ambient conditions, data at T = 0 h and T = 24 h from the pulse exposures are included in the ambient data. Ambient DO, pH, and temperature data are based on readings taken every 2–3 days; ambient ammonia data are based on weekly samples from treatments not being exposed to pulse or challenge conditions. DO values from pulse and challenge periods are based on readings taken ≈30–40 min apart during the exposure period. pH, temperature, and ammonia data were based on single readings taken as indicated, from replicate tanks on each treatment.

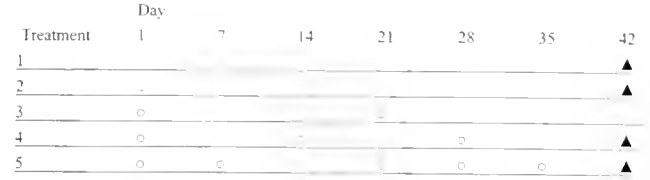

Figure 1. Pulse exposure regime for growth trial. ○ = pulse exposure. ▲ = challenge exposure. Animals were weighed and measured 2–3 days before the first exposure on day 1, and again on day 47. All animals were individually tagged. Treatment 1 is the control.

Water Quality

DO, pH, ammonia, and temperature for both pulse and challenge exposures were recorded before any alteration to conditions in the tank (T = 0 h). pH and ammonia were then measured after 15–20 min (T = 15 min), at 8 h (before restoring ambient conditions) and the morning following exposure (T = 24 h). Once DO had stabilized, it was checked on average every 30–40 min and flow of N_2 adjusted as required. DO, temperature, and pH were recorded for all tanks every 2–3 days throughout the trial. Samples for ammonia analysis were taken from two tanks on each treatment not being pulsed each week to provide ambient ammonia levels. DO was measured using hand- held meters (WTW Oxi96, TPS WP82-Y or Oxiguard Handy Gamma), which were calibrated daily and checked against saturated seawater and occasional Winkler titrations. The three meters gave similar readings when checked against each other. pH was recorded on a hand-held TPS meter and probe (WP 81), calibrated daily in fresh buffers (phosphate at pH 7.00, borate at pH 9.28, after Bruno & Svoronos (1989). Temperature was recorded using the thermistor on the DO meter, which was checked against a calibrated mercury thermometer.

All samples for ammonia analysis were collected in acid-washed glassware rinsed with deionised water. Samples were filtered through Whatman GF/C filters and frozen in polypropylene bottles (acid washed, rinsed in deionised water) for subsequent analysis. Total ammonia was measured within 4–5 wk of the samples being collected, by the method of Grasshoff (1989) but using the salicylate reagent of Bower and Holm-Hansen (1980). UIA was calculated from pH and temperature using the equation in Bower and Bidwell (1978). Where necessary, samples were diluted with the sand-filtered seawater such that TA was <0.5 mg L^{-1}. Nitrite was analyzed by the diazotization method of Grasshoff (1989). Influent flow rates were recorded every 5–10 days (mean ≈ 1500 mLmin^{-1}).

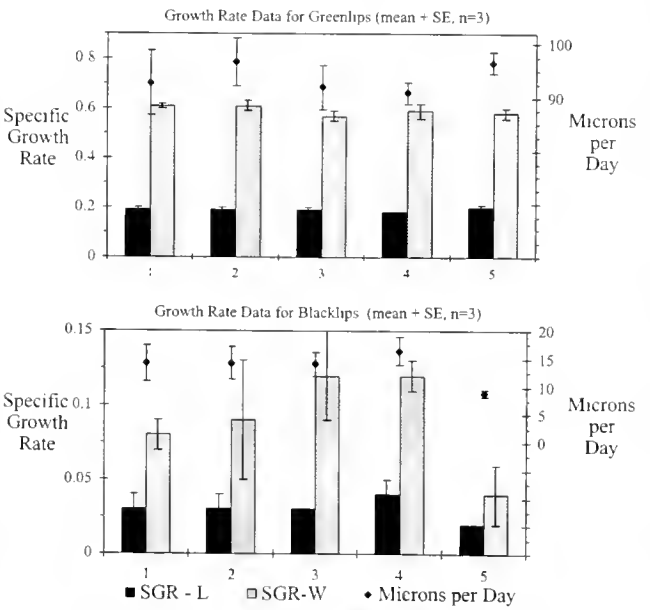

Figure 2. Growth rate data from pulse exposures of two species of abalone (mean ± SE, *n* = 3). Treatments are given in Figure 1. SGR-L = specific growth rate for length, *n* SGR-W = specific growth rate for weight.

Challenge Exposure

On day 45 of the growth trial, two tanks for each species on treatments 1, 2, 4, and 5 were exposed for 8 h to more severe challenge conditions (Table 1), as described above, but using a stock solution of 1.33 g L^{-1} of ammonia chloride.

Statistical Analysis

The raw data were tested for homogeneity of variance by examination of residual plots generated by JMP 3.2 (SAS Institute). Normality was assessed by observation of the distribution plotted by JMP 3.2. No transformations were required to meet the assumptions of analysis of variance (ANOVA), as given in Underwood (1981). One-way ANOVA, using the replicate means for each treatment, was used to look for significant treatment effects, and Tukey's HSD was used to compare means.

RESULTS

Behavioral Observations

Under ambient conditions, species-specific behavioral differences were observed. Blacklips clustered in one or two spots in the tank, but greenlip abalone tended to gather in smaller groups dispersed more evenly over the available surfaces. Although generally inactive, small numbers of blacklip abalone (1–3) were observed to be active at any one time during the day; whereas, greenlips were rarely observed to be active during the day. For both species, inactive animals had all tentacles withdrawn and shells clamped tightly onto the substrate. During the pulse exposures, no change in behavior was observed until the end of the exposure period, when signs of stress were evident (shells starting to lift off the substrate, tentacles becoming actively extended).

These behavioral effects were more pronounced during the final challenge exposure. In both species, the animals tended to separate from the clusters within 40–60 min of commencing the exposure, but once separated, did not continue moving. Rather, in both species, the front of the shell was lifted off the substrate, and epipodial tentacles were extended. However, abalone in this position were not readily removed from the substrate, and if disturbed, clamped the shell back onto the substrate and withdrew the tentacles.

Although food consumption was not directly determined by collecting uneaten food, ration was adjusted *ad libitum* for each tank, and no difference in weight of food fed between treatments was observed. However, in both species, consumption tended to decline slightly the night immediately after exposure to the pulse condition, and the abalone were also observed to be less active than normal. Consumption and foraging activity returned to normal the following night (24-h postexposure). Following the challenge exposure, this reduction in consumption and activity was

even more marked, but again returned to normal the following night.

Mortality

Over-all mortality was 0.8% for greenlips and 2% for blacklips during the growth trial. No mortality was observed as a direct result of the pulse or challenge exposures. There was no clinical evidence (such as lack of vigor, lesions, or mortality) of any secondary infection becoming established as a result of the pulse exposure regime. Two tanks of blacklips did show signs of bacterial infection at different points during the growth trial, with some mortality, although no causative agent could be identified. The other replicate tank on this treatment showed no similar signs, and these were considered unrelated events. These mortalities were excluded from the mortality calculations. Apart from these events, both species seemed generally healthy throughout the growth trial.

Water Quality

Table 1 shows the ambient water quality for days when DO and ammonia were not manipulated and the alterations achieved during pulses of ammonia and low DO. Table 1 also shows the water quality achieved during the final challenge exposure. Nitrite was always undetectable. The initial and final data collected for each pulse (at T = 0 h and T = 24 h) were always consistent with ambient conditions and, thus, were included in the ambient water quality.

Total ammonia was elevated throughout both the pulse and challenge exposure periods (Table 1). pH and UIA declined over the 8-h period, and DO was kept relatively constant by adjusting the gas flow. Temperature increased, to an extent dependent on ambient air temperature, by 1–5°C during the exposure periods. During the highest temperature increases, mucus production was evident, and the water became noticeably cloudy.

Growth Data

The growth data are shown in Figure 2. For greenlip abalone, growth rates (≈100 μm d^{-1}) were close to commercial growth rates. The blacklip abalone growth rates (≈15 μm d^{-1}) were approximately 10% of commercial growth rates. In terms of both length and weight, no significant difference was observed in growth of either species for any of the treatments ($P < 0.05$). For blacklip abalone, treatment 5 reduced growth, especially in terms of weight, although the difference was not significant.

DISCUSSION

The primary aim of this trial was to simulate a major systems failure, with an extended period of no influent water and little, if any, supplementary aeration, and the desired alterations in water quality were achieved within 5 min. This rapid change was designed to exacerbate the severity of the exposures further, because any similar changes during a real life event would take much longer to achieve. Although dissolved oxygen and ammonia were the primary water quality variables being manipulated, the lack of influent water flow also resulted in temperature increasing and pH declining over 8 h, which added to the reality of the simulation. Temperature changes reflected the ambient air temperature, which was beyond experimental control; whereas, the decline in pH was presumably attributable to respiration and metabolic excretions. Table 1 shows that substantially elevated TA levels were achieved over the baseline condition. Although TA levels did not change greatly during the exposure period, the decline in pH reduced the concentration of UIA.

It was considered possible that the water currents generated by the submersible pumps may have modified the response of the abalone to the stress induced by the pulses of poor quality water, perhaps by improving water movement past the gills. This is certainly consistent with known water-flow dynamics around and through abalone shells (Voltzow 1983), although animal behavior, in terms of both gross body positioning (Voltzow 1983) and underlying physiological modifications (see later), are also likely to be modifying factors. We are uncertain if this has been a significant factor in this trial, but note that at the end of the growth trial for Treatment 3, a pulse exposure was conducted without the submersible pumps, and no behavioral differences were observed from previous exposures (data not shown).

Based on observations of mortality and animal behavior, the pulse exposures and exposure regime used in this trial did not result in more than a transient stress. Coupled with the lack of significant effects on growth, this indicates that the two species used in this trial are well able to withstand periodic and relatively severe declines in water quality. Although a brief reduction in foraging activity was observed immediately following the pulse exposure, activity returned to normal the following night. The abalone seemed healthy throughout the growth trial, with the exception of an isolated event, and mortality was similar to previous trials in this system (e.g., Maguire et al. 1996). Conditions in the final challenge exposure seemed to be more stressful than the pulse exposures, based on observations of animal behavior, but were still not sufficient to produce mortality after 3 days. Consumption and activity patterns returned to normal within 24 h, as for the pulse exposures. The transient nature of the stress observed from the pulse and challenge exposures may have been at least partly attributable to optimal quality of the ambient conditions (Table 1).

Harris et al. (1998, 1999a) reported that greenlip abalone were more sensitive than other invertebrate species and fish when chronically exposed either to elevated UIA or nitrite or decreased DO. For DO and UIA, growth declined through the experimental range in these chronic studies (25–188 μg L^{-1} UIA and 8.9–4.2 mg L^{-1} DO (117–55% saturation)). However, these levels of UIA and DO were achieved in the pulse exposure from the current trial without producing a significant growth depression. Hindrum (unpublished data) also found that growth of blacklip and greenlip abalone was significantly reduced as compared to controls when chronically exposed to similar combinations of DO and UIA as achieved in the pulse exposures.

The chronic exposures outlined above were based on continuous exposure to altered water quality for several weeks. The pulse and challenge exposures in the current trial were for 8 h, during the normally inactive daytime period. It is likely that gill activity is reduced during this quiescent period. Certainly, for mollusks in general, this is the case, with activity levels, heart rate, gill perfusion rates, gill ventilation rates and resultant oxygen uptake intimately related. This has been shown in sedentary species like *Mytilus edulis* (Bayne 1971) and in different gastropod species exhibiting various survival/feeding strategies (Newell & Roy 1973, Morton 1990). Indeed, for abalone, temperature and external oxygen levels have been clearly linked to such factors as heart rate, blood pressure, and resultant oxygen uptake (Nimura & Yamakawa 1989, Russell & Evans 1989). In the quiescent period, tissue exposure to such toxicants as ammonia may be reduced as a

result of lower transfer across the gills and lower tissue perfusion rates. Indeed, low DO and the expected reduction in heart rate as seen for other haliotids (Nakanishi 1978, Russell & Evans 1989) may well have functioned in this way as a protective mechanism in our experiment. It is possible that a similar mechanism may also work in penaeids, as Allan and Maguire (1991) report that tiger prawns (*Penaeus monodon*) tolerated conditions for several hours that in chronic exposures resulted in significant growth reductions.

It is possible that a greater impact on growth may have been observed if the pulse exposures had been conducted during the nocturnal foraging period, or over a longer period (e.g., 12–24 h.). Potentially, the altered water quality would then have a bigger effect on the metabolic processes of the abalone, because they are active. However, the observed behavioral changes indicate that the altered water quality was still detected by the inactive abalone. The fact that exposing the abalone weekly to the pulse condition did not reduce growth or induce any clinical signs of stress, such as disease or morbidity, indicates that there was no additive effect. This is further confirmed by the lack of mortality and apparent recovery after the challenge exposure at the end of the growth trial.

The pH after 8 h of either challenge or pulse exposure was in the range 7.91–7.62. Harris et al. (1999b) found that chronic exposure to pH 7.76 significantly reduced growth in both species of abalone as compared to controls at pH 8.27. Although temperatures in individual tanks reached 22–23 degrees during periods of high ambient air temperature, the temperatures recorded after the 8 h of either pulse or challenge exposures were still within the tolerance limits defined by Gilroy and Edwards (1998) for these species. The mucus production observed in the current exposures on warmer days has been reported in abalone (Gilroy & Edwards 1998) and other bivalve mollusks (Bartsch et al. 2000) as a response to thermal stress.

Studies of haliotids in the wild show that these animals tend to be found in large groups, commonly stacked around boulders and in crevices at various depths (Shepherd 1973, Douros 1987, Shepherd & Partington 1995). *H. rubra*, in particular, occupies cave-like crevices during the quiescent daytime period in sufficient density that competition for space is a factor in their distribution (Shepherd 1973). In warmer weather and periods of low wave or tidal activity, low DO levels and elevated ammonia concentrations would be expected in these caves as a result of metabolic activity. This would be exacerbated when decaying organic matter accumulates (Wells et al. 1998). Not surprisingly, gastropod mollusks are physiologically and biochemically well suited to cope with episodes of hypoxia (Brix et al. 1979, Gäde & Ellington 1983, Storey & Storey 1990). The tolerance shown in this trial is, therefore, not unexpected.

CONCLUSION

Greenlip and blacklip abalone are resilient to periodic short-term exposure to low dissolved oxygen levels in conjunction with elevated ammonia levels. Transient effects on behavior and foraging activity were observed. Based on lack of mortality and on behavioral observations, exposure history had little effect on the response to a more severe challenge exposure. Evidence from the literature suggests that the low DO levels during the pulse and challenge exposures reduced tissue exposure to ammonia by reducing uptake across the gills.

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ONTOGENETIC TRENDS OF MINERALOGY AND ELEMENTS IN THE SHELL OF ABALONE, *HALIOTIS DISCUS HANNAI* INO

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ABSTRACT Japanese abalone, *Haliotis discus hannai*, with shell lengths 8, 14, 25, and 55 mm were used for mineralogy and element composition studies. Besides calcite and aragonite, dolomite ($CaMg(CO_3)_2$) was detected for the first time in abalone shells. No calcite was detected in the 8-mm shells. The percentage of calcite increased steadily from 1.6% to 13.6% in shells from 14 to 55 mm, while that of aragonite decreased from 95.3% to 83.9%. Dolomite varied from 2.5% to 9.0%. The level of Mg, Mn, Fe, Na, and Al in 8 to 55 mm shells increased, whereas those of Zn and Cu declined. Possible reasons for these trends are discussed.

KEY WORDS: ontogenetic trend, mineralogy, elements, abalone, *Haliotis discus hannai*

INTRODUCTION

The mineral composition of molluscan larval shells comprises exclusively of aragonite, regardless of postlarval or adult forms (Watabe 1988). In *Haliotis discus hannai*, the larval shells consist of aragonite spherulites (Iwata 1980), whereas the prismatic layer of adult shell is composed of calcite and aragonite (Dauphin et al. 1989, Shepherd et al. 1995). The changing pattern of shell mineralogy from larvae to adult remains unexplored.

Many factors that can influence the mineralogy and chemistry of molluscan shells have been investigated, including water temperature and salinity (Wilbur 1972, Taylor & Reid 1990, Cohen & Branch 1992, Mann 1992), taxonomic differences (Turekian & Armstrong 1960, Dauphin et al. 1989), ontogeny (Fuller 1985, Carriker et al. 1982, Carriker et al. 1982), and physiological conditions for shell formation (Simkiss 1976, Almeida et al. 1996). However, almost all of these studies investigated bivalves. Gastropods have been largely neglected. To our knowledge, there are no data on the ontogenetic trends of abalone shell mineralogy and chemistry.

In this study, the shells of different sizes of abalone, *Haliotis discus hannai*, were analyzed to study the ontogenetic trend of shell mineralogy and element composition.

MATERIALS AND METHODS

Abalone Shells

Live *H. discus hannai* individuals were purchased from Rongcheng Abalone Farm, China, in October 1998. These were cultured under the same environmental and nutritional conditions in the hatchery. Shells 8 ± 1 mm, 14 ± 1 mm, 25 ± 1 mm, 55 ± 1 mm (mean shell length ± s.d.) were used for this study. Upon sacrificing the abalone, their soft tissue was immediately removed.

X-ray Powder Diffraction

The mineralogical composition of the whole shell was investigated by X-ray powder diffraction. Ten to 20 shells were ground into a powder mixture for every replicate of each shell length. Three replicates of each shell length were used. A Philips counter diffractometer was employed, with a proportional counter and monochromatized copper target set at 40 kV and 80 mA. The ratio of polymorphs was calculated from the relative intensities of the principal reflection characteristic of each mineral through a calibration curve derived from mixtures of natural aragonite, calcite and dolomite (Neri et al. 1979).

Element Analysis

The abalone shells were ground into fine powder and carefully digested in double distilled nitric acid. Minor and trace elements in the digestion were analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) on a JOBIN (Model JY 70PLUS).

Statistical Analysis

All percentage data were square-root arcsine transformed prior to analysis. Tests of normality and homogeneity of variances confirmed that the data sets met the requirements for one-way ANOVA (Zar 1984). When overall differences were significant at the 5% level, Tukey's test was used to compare the mean values between individual treatments. Correlation analysis was employed to evaluate the relationship between abalone size and mineralogical or element composition.

RESULTS

Shell Mineralogy

The percentages of different crystal polymorphs detected for different shell lengths are given in Table 1 and Fig. 1. Dolomite was detected for the first time in abalone shells. Dolomite and aragonite were found in all samples. However, although expected, calcite was not detected in 8-mm shells. The percentage of calcite increased steadily from 1.6% to 13.6% in 14–55 mm shells, whereas that of aragonite decreased from 95.3% to 83.9%. Correlation analysis showed that the percentages of both calcite and aragonite were significantly correlated with abalone sizes (calcite: $r = 0.985$, $P < 0.0001$; aragonite: $r = -0.830$, $P < 0.001$). The proportion of dolomite increased from 4.7 to 9.0% in 8–25 mm shells, but dropped to 2.5% in 55-mm shells.

Element Composition

The element composition of different sized shells is listed in Table 2. Ontogenetic changes of selected elements differed. The levels of Mg, Mn, Fe, Na, Al in shells increased from 8 to 55-mm shells, whereas those of Zn and Cu dropped. The positive correlation between abalone size and the level of Fe ($r = 0.947$, $P < 0.0001$), Mg ($r = 0.769$, $P = 0.003$), Al ($r = 0.787$, $P = 0.002$),

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TABLE 1.

The mean percentage of different crystal polymorphs in abalone, *Haliotis discus hannai* Ino. of different shell length (n = 3, mean ± SD).

Shell length (mm)	Calcite (%)	Aragonite (%)	Dolomite (%)
8	n.d. (0.0)^a	95.3 (0.9)^b	4.7 (0.9)^a
14	1.6 (0.1)^a	93.3 (0.4)^c	5.1 (0.5)^a
25	6.6 (0.6)^c	84.5 (0.2)^a	9.0 (0.5)^b
55	13.6 (1.1)^b	83.9 (1.2)^a	2.5 (0.11)^a
ANOVA			
F	212.09	113.91	17.23
P	0.000	0.000	0.009

n.d.: not detectable

Means in the same row sharing the same superscript letter were not significantly different as determined by Tukey's test ($p > 0.05$).

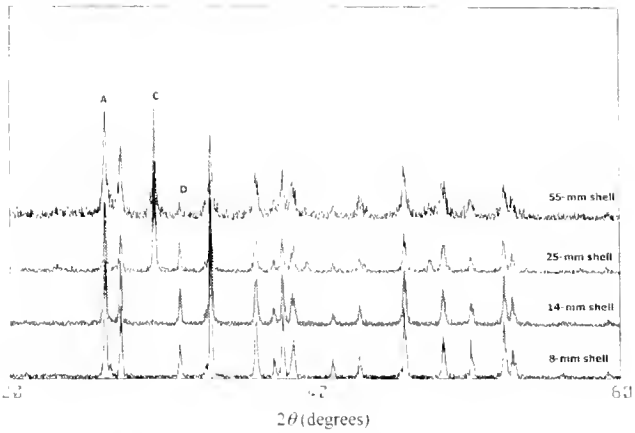

Figure 1. X-ray diffraction profiles of different *Haliotis discus hannai* shell sizes. Main reflections specific to aragonite (peak labeled 'A'), calcite (peak labeled 'C'), and dolomite (peak labeled 'D') are indicated.

or Na (r = 0.612, P = 0.034) was significant, as were the negative correlation between abalone size and Zn (r = −0.721, P = 0.008) and Cu concentrations (r = −0.646, P = 0.023). No significant correlation was established between abalone size and the level of Mn (r = 0.480, P = 0.114).

DISCUSSION

It has been reported that the shell mineralogy of adult and larval shells is quite different (Watabe 1988), although to date this had never been investigated in abalone. This is an important avenue of research, however, as the ontogenetic change of shell structure can determine the hardness of the shell, and thus affect the survival of a mollusk since its shell is usually its main source of defense.

In our experiments, clear ontogenetic trends of mineralogy and element composition were observed. The definite intrinsic mechanisms that determine the mineral composition of shells during ontogenetic development remain unknown, although several factors are known to be involved in the process. In *in vitro* tests, matrix proteins secreted by mantle epithelial cells determine the biomineral polymorphs of shells. Macromolecules extracted from the aragonite layers of some mollusk species induced aragonite formation *in vitro*, and macromolecules from calcite layers induced mainly calcite formation under the same conditions (Belcher et al. 1996, Falini et al. 1996). It has been reported that the composition of matrix proteins of abalone shell varied in different developmental stages (Cariolou & Morse 1988). We obtained similar results in our laboratory (unpublished data). Therefore, we suggest that the matrix proteins can play a vital role in controlling the composition of crystal polymorphs in shells. Further work is needed to characterize proteins in different shell layers during ontogenetic development and relate their composition to the crystal formation. On the other hand, some environmental factors, such as water temperature and salinity can also influence shell mineralogy (Taylor & Reid 1990, Cohen & Branch 1992). However, given the constant environmental conditions (e.g. water temperature, salinity and nutritional conditions) in our hatchery, the influences of these factors on our results can be largely excluded.

Unexpectedly, besides calcite and aragonite, dolomite was detected in the current study. The mechanism of its formation remains unknown. It is reported that the arrangement of atoms in aragonitic and calcitic lattice structures allows for the substitution of some divalents for calcium ions (Rosenberg 1990, Lingard et al. 1992). Under certain circumstances, this substitution can even result in separate crystal polymorphs. Several isomorphs, such as vaterite (Wilbur & Watabe 1963), strontianite ($SrCO_3$) (Odum 1951), dahllite (Watabe 1956) and barite (Fritz et al. 1990) have been reported. Pytkowicz (1965) points out that under conditions in which the Mg concentration is five times that of Ca, which is common in sea water and some bivalve extrapallial fluids (Crenshaw 1972, Wada & Fujinuki 1976), collisions producing Mg-Ca-CO_3 aggregates are more frequent than those resulting in $CaCO_3$.

TABLE 2.

Mean concentration (ppm) of certain elements in abalone, *Haliotis discus hannai* Ino. of different shell length (n = 3, mean ± SD).

	Shell length (mm)				ANOVA	
Element	8	14	25	55	F	P
Zn	27.2 (0.0)^b	53.1 (6.0)^c	6.9 (0.2)^a	0.2 (0.2)^a	123.89	0.000
Mn	4.7 (0.0)^a	1.7 (0.1)^a	16.4 (1.4)^b	10.5 (3.0)^b	44.12	0.002
Fe	28.9 (2.0)^a	29.6 (3.5)^a	133.8 (28.4)^b	211.9 (1.3)^c	76.08	0.006
Mg	140.1 (0.7)^a	119.2 (6.8)^a	386.8 (22.4)^b	373.7 (0.5)^b	305.28	0.000
Cu	6.2 (0.2)	9.1 (4.0)	2.7 (0.3)	2.5 (0.2)	3.63	0.122
Al	1498.8 (10.6)	1373.3 (30.3)	1344.4 (44.5)	1785.2 (36.1)	4.93	0.079
Na	6883.6 (11.7)^a	9527.2 (96.1)^b	10439.1 (24.6)^b	10064.6 (74.5)^b	20.21	0.007

Means in the same line sharing the same superscript letter were not significantly different as determined by Tukey's test ($p > 0.05$).

There is still no report regarding the concentration of these ions in abalone extrapallial fluid (EPF).

This study reports that the levels of Mg, Mn, Fe, Na, and Al in shells increased with shell size (age), whereas those of Zn and Cu dropped. This is similar to what is reported by Carriker et al. (1982) concerning the oyster *Crassostrea virginica* (Gmelin). These changes can be attributed to many factors. For example, magnesium and manganese are enriched in calcite rather than aragonite (Dauphin et al. 1989). Mn can accumulate during the process of shell thickening (Yoshioka & Terai 1996). Hawkes et al. (1996) reported that the concentrations of elements in the shell were very different in the outer prismatic shell layer and the inner nacre layer. Therefore, the concentration changes of elements can be regarded as the effects of changes of shell mineralogy during ontogenetic development.

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GROWTH OF JUVENILE ABALONE, *HALIOTIS FULGENS* PHILIPPI, FED DIFFERENT DIETS

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ABSTRACT Growth rates of juvenile *Haliotis fulgens* (green abalone) were evaluated with four different diets over 106 days. Three diets were based on the algae palm kelp *Eisenia arborea*, giant kelp *Macrocystis pyrifera*, and *Gelidium robustum*, and one on seagrass *Phyllospadix torreyi*. One artificial diet was used as a control. The best growth rates and specific growth rates were found in abalone fed *M. pyrifera*, which were significantly different from growth achieved on the other natural diets. The pattern of growth in juveniles fed an artificial diet was similar to juveniles fed *M. pyrifera*. The highest mortality (11%) was in juveniles fed the red algae *G. robustum*.

KEY WORDS: *Haliotis fulgens*, green abalone, growth, algae, diets

INTRODUCTION

In recent years, dietary research in abalone has focused on the production of formulated feeds in countries with a history of abalone culture (Uki & Watanabe 1992, Wee et al. 1992, Viana et al. 1993, Viana et al. 1994, Britz 1996a, Britz 1996b, Fleming et al. 1996, Knauer et al. 1996, Britz et al. 1997, Clarke & Creese 1998, López et al. 1998, Monje & Viana 1998) because of potential use of these feeds in commercial production. Information on natural diets is important in understanding the biology of abalone species. Abalone food preferences have been studied from both analysis of gut contents and feeding experiments in species such as *Haliotis cracherodii* Leach, *H. discus hannai* Ino, *H. fulgens* Philippi, *H. tuberculata* (L.), and *H. rufescens* Swainson (Sakai 1962, Leighton & Boolootian 1963, Culley & Peck 1981, Uki 1981, Uki et al. 1986, Leighton 1966, Mercer et al. 1993, Corazani & Illanes 1998, Leighton & Peterson 1998, Simpson & Cook 1998). Shepherd and Steinberg (1992) reviewed the literature on the feeding biology of abalone. In Mexico, research into natural diets has been limited to the brown alga *Macrocystis pyrifera* (L.) C. Ag. as a control in feeding trials (Viana et al. 1993, Viana et al. 1996). It has been assumed that abalone feed on the kelp alga *M. pyrifera*. Regional hatcheries use this species as a main source of natural food. *M. pyrifera* does not occur at the southern limit of the distribution of abalone species along the Baja California Peninsula. However, there are many subtidal algae along the coast that may be used as potential food. In Baja California Sur, the most common food items in gut content of adults of green abalone *Haliotis fulgens* were the seagrass *Phyllospadix torreyi* S. Watson and the macroalgae *Sargasum* sp., *Eisenia arborea* Aresh., *Cryptopleura crispa* Kylin, and *Rhodymenia* sp. (Serviere-Zaragoza et al. 1998). It is important to develop feeding experiments oriented to test growth rates and feed conversion efficiencies on single species diets to evaluate local algal species as diets for abalone. Day and Fleming (1992) mentioned that although an alga may not support sustained growth when fed alone, it may be of great value when part of a mixed diet by providing essential nutrients to the diet. This study was designed to assess the growth of juvenile green abalone fed with common species in the benthic environments inhabited by abalone along the western coast of Baja California.

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MATERIALS AND METHODS

Diets

Macrocystis pyrifera (MP) was selected as the primary alga species (the control) because it is the dominant algal species of southern California (Dawson et al. 1960) and it is believed by fishermen that Mexican abalone species feed extensively on this alga. The brown alga *Eisenia arborea* (EA), red alga *Gelidium robustum* (GR), and the sea grass *Phyllospadix torreyi* (PT) are thought to be important species in abalone communities as potential food along Baja California (Guzmán del Próo et al. 1972, Guzmán del Próo et al. 1991, Serviere-Zaragoza et al. 1998). Natural diets were harvested, dried, and stored at the beginning of the experiment, because chemical composition varies during the year. The growth rate of abalone fed with algal diets is low and variable over time. Therefore, we decided to include an artificial diet as a control (AD). AD was manufactured by the nutrition group of Instituto de Investigaciones Oceanológicas, B.C. The dietary formulation is in Table 1. Proximate analyses (crude protein, ether extractables, crude fiber, and ash) were performed using the methods of the Association of Official Agricultural Chemists (1995).

Experimental Procedure

Animals used in this experiment were reared in a commercial hatchery in Eréndira, Baja California, and transferred to the CIBNOR laboratory in La Paz, B.C.S. at age 8 mo. Feeding experiments commenced six months later. During this period, animals were fed the brown alga *Eisenia arborea*. Six hundred animals (17.3 (±2.2) mm shell length and 0.44 (±0.2) g body weight) were used to test the growth response of abalone fed four diets for 106 days. Experimental animals were held in 16-L fiber glass containers (50 × 30 × 35 cm, experimental units) with a concave bottom. Three replicates per treatment were made with 40 abalone per experimental unit (EU). Animals were marked with plastic tags attached to the shell. The EUs were supplied with temperature controlled (20 ± 1°C), fresh, filtered (10 μm) water with a flow rate of 73 mL/min. The water was aerated vigorously. Salinity, oxygen, pH, nitrate, nitrite, ammonium content, and phosphate were monitored every week. Dead animals were removed and replaced to maintain densities. Shell length was measured with a vernier caliper, and body weight was measured with an electronic balance (to 0.001 g) at the beginning, and at 30, 60, and 106 days.

TABLE 1.

Percent composition of the artificial diets tested in this study, given as percentage of dry matter.

Ingredients	g/100 g
Fish meal	30.0
Silage (dry basis)	2.0
Soy bean meal	10.0
Corn meal (whole)	12.0
Vegetable meal	15.0
Corn starch	19.37
Gelatin	6.0
Vitamin mixture	1.7
Mineral mixture	3.3
Choline chloride	0.11
Methionine	0.23
BTH	0.086
Sodium Benzoate	0.23

Diets were given *ad libitum*. Natural diets were fed every 42 hours in the afternoon and the artificial diet each night. The remaining food was carefully collected for drying and weighing; every other morning for natural diets and each morning for the artificial diet. This was undertaken throughout the feeding experiment. Algae growing on the inside walls of the EU were removed twice a week with a soft brush. For diets, the amount of dry matter lost in seawater was estimated during the experiment by using EUs without abalone under the same conditions as those of the growth experiments.

Growth rates were calculated by the equation:

$$GR_{SL} = (SL_f - SL_i)/T \text{ and } GR_{BW} = (BW_f - BW_i)/T$$

where SL_f = mean final shell length, SL_i = mean initial shell length, BW_f = mean final weight, BW_i = mean initial weight, and T = time in days.

Specific growth rate (SGR % day^{-1}) was calculated for shell length and body weight by the equation (Britz 1996b):

$$SGR_{SL} = \{(\ln SL_f - \ln SL_i)/T\} \times 100 \text{ and}$$

$$SGR_{BW} = \{(\ln BW_f - \ln BW_i)/T\} \times 100$$

where SGR_{SL} is percent shell length gain per day, SL_f = mean final shell length, SL_i = mean initial shell length, and T is time in days between measurements. SGR_{BW} is percent body weight gained per day, BW_f = mean final weight, and BW_i = mean initial weight.

Consumption was calculated in terms of dry weight with the equation (Uki & Watanabe 1992):

$$FC = (GS/100) - R$$

where G is the weight of food offered per animal per day (in grams), S is the percentage of food recovered, obtaining a factor for each diet (from the controls without abalone), and R is the remaining food (in grams) after the abalone had fed.

Food conversion efficiency ratio was calculated as (Uki & Watanabe 1992):

$$FCE = \text{wet weight gain (g)/dry weight of food consumed (g)} \times 100$$

This measure of food utilization is related to conversion efficiency, but is in fact the ratio of animal live or wet weight gain to the amount of dry diet consumed (Monje & Viana 1998).

Statistical Analyses

Data for experimental replicates were pooled because no significant differences were found between them by one-way analysis of variance (ANOVA) at a significance level of $P = 0.05$. Data from each different treatment were analyzed by ANOVA test and Tukey test to determine differences of means (Sokal & Rohlf 1995). Statistical analysis was done with software STATISTICA 6.0 for PC.

RESULTS

The highest protein content was found in *Gelidium robustum* (GR) followed by *Phyllospadix torreyi* (PT), *Macrocystis pyrifera* (MP), and *Eisenia arborea* (EA). The lipids were between 1.06% for *G. robustum* and 1.30% for *M. pyrifera* (Table 2). The daily average temperature was 20 ± 1°C. Water quality analyses showed the following averages (±*s*) for pH 8.03 (±0.06); oxygen 6.52 (±0.74) mg/L, salinity 40 (±1.29) ppm, nitrites 0.0052 (±0.0004) µmol/L, nitrates 0.1253 (±0.0029) µmol/L, ammonium 0.0226 (±0.00001) mg/L, and phosphate 0.00552 (±0.000017) µmol/L throughout the experiment.

Growth of Abalone

A significant difference in means was shown between shell length and body weight of juvenile green abalone fed with the natural diets ($P < 0.05$). At 30 days of the experiment, the mean length, 19.15 mm ± 0.23 (SE), and weight, 0.59 g ± 0.02 (SE), of juveniles fed MP were statistically different from the means of juveniles fed EA, 18.28 mm ± 0.21 (SE) and 0.02 g ± 0.18 (SE), and GR, 18.24 mm ± 0.19 (SE) and 0.50 g ± 0.02 (SE). After 60 days, the differences increased and were significant. Both mean length and weight of juveniles fed MP were statistically different from the other diets; EA, GR, and PT. Differences between juveniles fed these latter three diets were not detected.

Mean shell length and body weight increased over time on all diets (Fig. 1). The best growth in length and weight for green abalone was obtained with MP, 22.29 mm ± 0.33 (SE) and 1.0 g ± 0.06 (SE). The percent of survival was between 89% and 95% in natural diets. For AD, it was 97% (Table 3). The pattern of growth in juveniles fed the artificial diet, 22.01 mm ± 0.22 (SE) and 0.91 g ± 0.03 (SE), was similar to juveniles fed MP (Fig. 1).

TABLE 2.

Proximate analysis of the species and artificial diet used in experimental diets. EA, *Eisenia arborea;* MP, *Macrocystis pyrifera;* GR, *Gelidium robustum;* PT, *Phyllospadix torreyi;* and AD, artificial diet.

	Component				
Diet	Crude Protein	Ash	Crude Fiber	Ether Extract	N-free Extract
EA	7.60	27.13	6.44	1.15	57.68
MP	12.0	41.33	7.0	1.30	38.37
GR	17.61	21.26	10.19	1.06	49.88
PT	15.94	31.88	13.45	1.28	37.44
AD	35.85	10.70	5.85	7.09	40.51

Values are given as percent of dry matter.

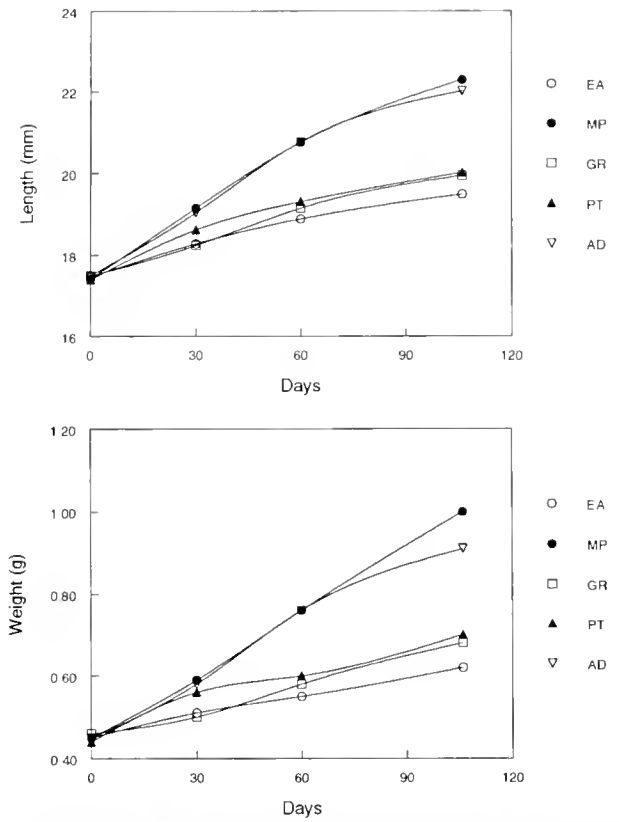

Figure 1. Mean growth of abalone fed with different diets. EA, *Eisenia arborea;* MP, *Macrocystis pyrifera;* GR, *Gelidium robustum;* PT, *Phyllospadix torreyi;* and AD, artificial diet.

Growth Rates

Significant differences occurred, between natural diets, in the daily growth rates of the shell length (SL) and body weight (BW) ($P < 0.05$). During the experimental period, 106 days, both mean SL and BW growth rates of animals fed MP were higher than mean growth rates of the juveniles fed the other natural diets ($P < 0.05$) (Table 3). The growth rates of the juveniles analyzed varied within the same diet during the experiment. In EA and PT a gradual decrease was observed during the experiment. For MP and GR, in the second month a gradual increase was measured, and at the end of the experiment the values had decreased. Juveniles fed EA showed the lowest shell length growth rates, 15 μm ± 1 (SE), during the third experimental month and the highest was in juveniles fed MP, 53 μm ± 2 (SE), during the second month. Body weight growth rates were between 0.92 mg ± 0.13 (SE) for GR during the first month and 6.70 mg ± 1.29 (SE) for MP during the third month.

The daily growth rates of juveniles fed artificial diet were similar to growth rates of juveniles fed MP (Table 3). The values ranged between 31 μm ± 2 (SE) in the third month to 57 μm ± 2 (SE) in the second month. Daily body weight rates were between 3.63 mg ± 0.18 (SE) in the first month and 6.13 mg ± 0.23 (SE) in the second month.

SGR

The ANOVA showed that there was no significant difference in mean SGR for any of the replicate diets. Using the Tukey test on the SGR (Tukey test $P < 0.05$) for green abalone, the existence of a significant difference in mean was shown between the SGR shell length and body weight of the abalone fed with MP and the other diets (EA, GR, PT). The same analysis revealed there was no significant difference in the mean SGR for EA, GR, and PT, except for SGR body weight of juveniles fed PT, which was higher than EA and GR (Fig. 2). SGRs obtained from juveniles fed the artificial diet were similar to juveniles fed MP.

The feed consumption rate of abalone ranged from 0.0033 g/day for EA to 0.0108 g/day for MP. Consumption did not differ significantly between EA and GR, and PT and MP ($P > 0.05$). Abalone consumed significantly greater amounts of PT and MP (Table 4). FCE ratios for the natural diets vary between 30% for PT and 63% for MP (Table 4). Differences between FCE ratios were not detected ($P < 0.05$).

DISCUSSION

Mean shell length and body weight increased over time on all diets. The best growth in length and weight for green abalone was obtained with *Macrocystis pyrifera.* Feeding abalone on *Eisenia arborea, Gelidium robustum,* and *Phyllospadix torreyi* diets resulted in lower growth and FCE, 46% to 81% of the values obtained with *M. pyrifera.* These results indicate that the dietary value of the common species along the coast of Baja California Sur, *Eisenia arborea, Gelidium robustum,* and *Phyllospadix torreyi,* was inferior to that of the dominant algal species of southern California *M. pyrifera.* This result may be related to the trends described by Guzmán del Próo et al. (1976) about the size and weight means for *Haliotis* spp., which decrease from north to south along the Baja California Peninsula. In southern California, the

TABLE 3.

Survival, mean initial size, mean growth gain, and mean growth rate of green abalone fed with different diets. Diets as defined in Table 2.

Diet	Survival %	Mean Initial Size (mm)	Mean Growth Gain (mm)	Mean Growth Rate (μm day^{-1})	Mean Initial Size (g)	Mean Growth Gain (g)	Mean Growth Rate (mg day^{-1})
EA	93	17.47 (0.20)	1.91 (0.10)	19 (0.89)^a	0.45 (0.01)	0.15 (0.01)	1.52 (0.09)^a
MP	93	17.42 (0.20)	4.63 (0.23)	46 (1.96)^b	0.45 (0.01)	0.55 (0.06)	5.49 (0.54)^b
GR	89	17.49 (0.21)	2.22 (0.21)	23 (1.94)^a	0.46 (0.01)	0.20 (0.02)	2.07 (0.19)^a
PT	95	17.40 (0.20)	2.52 (0.11)	25 (0.95)^a	0.44 (0.01)	0.25 (0.01)	2.43 (0.10)^a
AD	97	17.50 (0.17)	4.47 (0.13)	42 (1.21)^b	0.45 (0.01)	0.46 (0.02)	4.39 (0.24)^b

Standard error in parentheses.

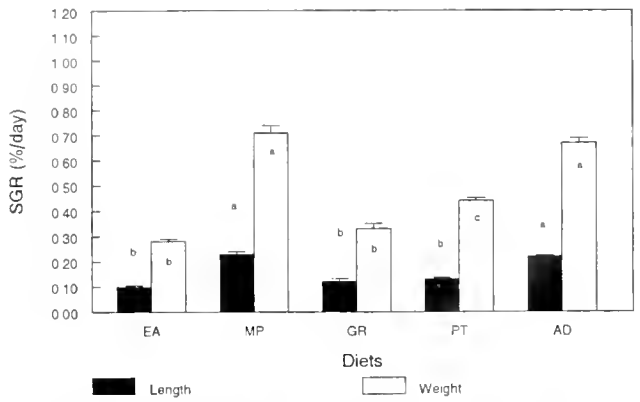

Figure 2. Mean specific growth of abalone compared between diets. Diets as defined in Figure 1.

TABLE 4.

Consumption (FC) and Food conversion efficiency (FCE) of green abalone fed with different diets. Diets as defined in Table 2.

Diet	FC (g)	FCE (%)
EA	0.0033 (0.0002)^{a}	52 (5)
MP	0.0108 (0.0010)^{c}	63 (16)
GR	0.0043 (0.0009)^{ab}	33 (.01)
PT	0.0102 (0.0009)^{c}	30 (7)
AD	0.0075 (0.0004)^{b}	59 (2)

Standard error in parenthesis.
Items with different superscript letters are significantly different.

best growth rates for juveniles and young adults have been observed when the alariacean brown alga, *Egregia menziesii*, served as food. The giant kelp, *Macrocystis pyrifera*, is of relatively minor value as a food of green abalone (Leighton & Peterson 1998). *E. menziesii* does not occur in the natural habitats of the different abalone species in southern Baja California (Guzmán del Próo et al. 1991). Local studies oriented to evaluate common algae along southern Baja California as a diet of abalone are of interest in the knowledge of abalone biology and for abalone hatcheries developed along this area of the coast. Fishermen have been forced to develop abalone hatcheries to produce juveniles and to increase natural stock. Hatcheries are going to use microalgae and macroalgae as the diet for juveniles until an artificial diet is successfully developed.

The nutritional value of food rations depends on many factors, including nutrient composition, bioavailability, palatability, and digestibility. Food palatability is an important factor in determining feeding rates (Leighton & Boolootian 1963, Leighton 1966, Poore 1972, Fleming 1995). In this study, the difference in growth between juveniles fed MP and EA or GR might be caused mainly by differences in palatability because of the lower amount consumed using both EA and GR. Differences between growth of juveniles fed MP and PT might be related to the digestibility of the protein sources because of the lower FCE of the seagrass. Poor abalone growth rates observed for seaweed diets could be attributed to a deficiency of essential nutrients or a low protein to energy ratio, because marine algae, in general, are rich in storage carbohydrates but low in protein. Thus the abalone fed macroalgal diets may satisfy their energetic requirements primarily from carbohydrate, but sufficient protein may not be available for tissue deposition (Britz 1996b). Beside variables associated with the food quality, digestion of the food is an important issue in nutrition. Our group hereby describes the enzymes responsible for digestion of this protein found in the organism's digestive system. In the adult green abalone trypsin and chymotrypsin activity was found in both intestine and rectum, but not in hepatopancreas and crop-stomach content. In juvenile green abalone digestive extracts revealed hepatopancreas and viscera hydrolyzed trypsin, chymotrypsin, and acid phosphatase specific substrates. (Serviere-Zaragoza et al. 1997, Picos-Garcia et al. 2000).

The growth rate of juvenile green abalone was slow and heterogeneous. The average growth rates of the juveniles analyzed varies from 19 μm day^{-1} and 1.52 mg day^{-1} for *Eisenia arborea* to 46 μm day^{-1} and 5.49 mg day^{-1} for *M. pyrifera*. Viana et al. (1993, 1996) reported averages of 12 μm day^{-1} and 16 μm day^{-1} for juveniles fed fresh kelp, *M. pyrifera*, and 18 μm day^{-1} for juveniles fed an artificial diet based on kelp meal for the same abalone species. *Gelidium robustum*, *Eisenia arborea*, and *Phyllospadix* spp. are of low acceptability for green abalone in Southern California and support minimal growth (Leighton pers. comm.). Simpson and Cook (1998) found shell length growth rates of *H. midae* ranged between 15 and 53 μm day^{-1} on single-species diets of *Ecklonia*, *Laminaria*, *Porphyra*, *Ulva*, *Aeodes*, and *Gracilaria*.

Using EA and PT, a gradual decrease was observed during the experiment. For MP and GR in the second month, a gradual increase was observed, and at the end of the experiment the values had decreased. This is similar to the trend reported by Viana et al. (1993); the daily growth rates decreased throughout time. Feeding trials on *H. rubra* using single species of dried algae revealed that abalone cease to grow after a period ranging from 50 to 200 days (Day & Fleming 1992). In the wild, abalone species consume more than one species. In *H. fulgens* the average number of plant species per gut was between two and four (Serviere-Zaragoza et al. 1998). This suggests they obtain the required nutrients for growth from combinations of species.

An artificial diet provides better growth rates than a natural one (macroalgae) in abalone cultures (Nie et al. 1986, Hahn 1989, Uki & Watanabe 1992, Viana et al. 1993, Viana et al. 1996). Nevertheless, in this study the growth of juveniles fed the artificial diet used as a control was similar to growth of juveniles fed MP. The lower growth of juveniles fed AD may be related to the leaching of some components during the transport or storage of the artificial diet. All environmental variables were constant during the experiment with abalone strongly attached and active throughout. Additionally, growth rates in juveniles fed MP were similar to that reported in the literature for the same species (Viana et al. 1993, Viana et al.1996).

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IDENTIFICATION OF EXPRESSED HSP'S IN BLACKLIP ABALONE (*HALIOTIS RUBRA* LEACH) DURING HEAT AND SALINITY STRESSES

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ABSTRACT Both prokaryotes and eukaryotes express a set of highly conserved proteins in response to external and internal stress. The stressors include tissue trauma, anoxia, heavy metal toxicity, infection, changed salinity, and the most characterized, heat shock. The result is an expression of stress proteins or heat shock proteins (HSP's) which lead to protection of protein integrity, and also to tolerance under continued heat stress conditions. The Australian blacklip abalone (*Haliotis rubra*) is found principally in southern coastal waters and also in estuarine/bay environments. Estuarine/bay environments have greater fluctuations in environmental conditions, especially those of salinity and water temperature, than are found along oceanic coasts. Abalone from estuarine/bay and oceanic coastal environments were subjected to either increased temperature (2°C/day for a total of 10°C) or hyposalinity (80% seawater). Estuarine/bay abalone were less affected than the oceanic animals by temperature increase and also demonstrated the ability to volume regulate 3 h after the initial salinity shock. SDS-PAGE and Western blotting techniques, together with dot blots of total protein, using HSP70 specific antibodies, were used to detect HSP70s in the foot muscle of the animals and indicated an expression of HSP70 in response to heat shock in abalone, but not following hyposalinity shock. RT-PCR yielded a partial cDNA clone of HSP70 from the foot muscle.

KEY WORDS: abalone, *Haliotis rubra*, heat shock proteins, HSP70, stress, cDNA

INTRODUCTION

Extreme environmental conditions cause all organisms previously studied, from mammals to bacteria, to evoke a heat shock response (HSR) (Gehring & Wehner 1995). The HSR is a rapid reaction to environmental or internal stresses that can be elicited by factors such as tissue trauma, mutagens, anoxia, heavy metals, salinity and exposure to abnormally high temperatures (Laursen et al. 1997). The HSR is more generally known as the stress response, because previous studies have shown that a wide range of stressful conditions induce its initiation. One exception to date has been established, *Hydra oligactis*, a freshwater Cnidarian that lives in thermally stable environments (Bosch et al. 1988).

Following stress, the HSR leads to significant alterations in expression of a small number of specific genes in the organism, designated as heat shock genes. These genes become actively transcribed under stressed conditions, which in turn leads to production of certain proteins, aptly named heat shock proteins (HSPs). The name is derived from the original stressor which led to the discovery of these proteins (Gehring & Wehner 1995). Upregulation in the transcription of one of these HSPs, HSP70, during stress is thought to be due to an elevation in the cellular levels of denatured proteins that activate a transcription factor termed the heat shock factor (HSF), which in turn binds to GC rich DNA in the promoter regions upstream to the HSP70 gene (Sanders 1993). Upon binding of HSF the upregulation of HSP70 expression is possibly 100 times basal levels (Wilkins & Lis 1997). The significance of the HSR is only partially understood, but there is sufficient evidence to indicate that production of HSPs leads to protection or tolerance of the stress to which the organism is exposed (Wood et al. 1998).

HSP70s are among the most highly conserved family of proteins known. There is approximately 50% homology in the amino acid (AA) sequence of every species characterised (Parsell & Linquist 1994). This high homology suggests there has been little phylogenic divergence in the protein, highlighting the importance of HSPs to survival. Most of the highly conserved regions in HSPs are found in the amino-terminus (N-term) of the protein, where an ATP binding domain is located. The carboxy-terminus (C-term) is the more divergent as it contains the substrate binding domains that recognize the vast array of proteins.

There are two distinct types of proteins in the 70kDa class, the inducible HSP70 proteins and non-inducible (constitutive) heat shock cognates, HSC70s (Wood et al. 1998). HSC70s are important in the normal function of a cell under non-stressed conditions and therefore are present at detectable low levels under such conditions. HSC70 proteins are important in the translocation of proteins within cells and especially in the transport of proteins into the mitochondrion (Stuart et al. 1994). HSC70s also prevent folding of polypeptide chains by cradling them when incorrectly folded or not completely assembled. Such a mechanism prevents the appearance of nonsense proteins and the aggregation of unfolded AA chains, which leads to cellular damage.

Earlier observations on HSPs were interspecies comparisons in expression, or differences between HSP expression in control and stressed animals of the same species. These demonstrated variations in the protein expression in both cases. For example, the lamprey *Lampetra appendix* has a HSP induction temperature (26°C), which is approximately 4°C higher than that of *Petromyzon marinus*, another lamprey species (Wood et al. 1998). Such differences are believed to be a consequence of evolutionary histories that ultimately led to genetic differences in the heat shock response. In another study, HSP expression was observed in two Californian mussel species, *Mytilus trossulus* and *Mytilus galloprovincialis* (Hofman & Somero 1996). Different latitudes separate these species, *M. trossulus* being the more northern of the two. *M. trossulus*, was the more thermally sensitive, as measured by HSP70 standing-stocks and ubiquitin conjugates present in the gill tissues of animals maintained at 13°C for 8 wk. In addition, induction temperatures for new HSP70 synthesis were lower in *M. trossulus* than in M. *galloprovincialis* (23°C versus 25°C), suggesting protein damage at lower temperatures and greater temperature sensitivity in the species from the cooler climate. More recently, Chen and Chen (1999) have shown that juvenile abalone acclimated to higher temperatures survived heat shock at higher

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temperatures. Interestingly, these abalone heat shock responses were negatively correlated with salinity.

Salinity stress is another factor encountered by coastal marine organisms. *Eurytemora affinis*, a crustacean found in estuaries of North America and Europe, expresses a range of proteins when exposed to a salinity gradient, with particular groups of proteins being expressed at different salinities (Gonzalez & Bradley 1994).

Molecular evidence for tolerance to heat has been characterised in a molluscan cell line from *Biomphalaria glabrata*, a fresh water snail (Laursen et al. 1997). Western blotting showed increased expression of HSP70 resulting from a 10°C increase. Northern blots and RT-PCR led to the cloning of an induced HSP70 cDNA in this snail, which was sequenced and compared to that of other organisms. The highest sequence homology was to that of HSP70s of other aquatic animals, including HSP70a and HSP70b in *Aplysia californica*.

Abalone (genus *Haliotis*) is a marine mollusc which inhabits coastal and bay waters of southern Australia. During summer, bay abalone have been observed to dramatically lose weight and deteriorate in condition. Factors causing such deterioration could include the hotter seawaters (e.g. bay 10°C greater than ocean) and fluctuating salinity during periods of high rainfall. Therefore, we started the current study examining the HSR in commercially important blacklip abalone, *Haliotis rubra* (Leach) exposed to elevated temperatures. In addition, we compared the ability of bay and oceanic populations to volume regulate after a hyposaline challenge.

MATERIALS AND METHODS

Animal Collection and Maintenance

Haliotis rubra (Leach) were collected during Spring under permit number 97/R/049A from a northern Port Phillip Bay site near Point Cook and from a site in ocean water near Barwon Heads in Victoria, Australia. The animals were transported to aerated holding tanks with a fresh filtered flow-through seawater system where ambient water temperature ranged from 12–16°C. They were provided an excess diet of mixed red macroalgae. The animals were held for 2–4 wk before experimentation.

Environmental Manipulation: Salinity Stress

Two replicate hyposalinity tests took place in two 36-L tanks in which 80% sea water was produced from sea water taken from the holding tanks and distilled water. Water temperatures of the two replicate tanks were 16 ± 0.2°C for test 1 and 13.5 ± 0.2°C for test 2, being the ambient temperatures at the times of experimentation. Plastic plates of 300 mm × 300 mm were weighed wet, numbered, and placed vertically in the tanks. Six abalone were taken from each population, weighed, measured by shell length, and then placed on plates in the tanks (one population per tank). No water flow was permitted during the salinity trials and a carbon fiber filter and aerator were placed in each tank to provide sufficient oxygen and remove any organic matter the abalone might produce. The animals were weighed at 0.5 hour on the plates to reduce disturbance, and then weighed (on the same plate each time) at 1 h intervals for 6 h. Weights were also measured at 24 h, 48 h and 72 h. Abalone were returned to their respective holding tanks after the 72 h test period had finished. No animal was used more than once.

Additional abalone were treated in the same manner for 1.5 h before taking tissues for molecular studies.

Environmental Manipulation: Heat Stress

Responses to heat stress were examined in a 90-L glass tank in which 15 abalone were taken at random from the bay and ocean populations. The critical thermal maximum (CTM) was determined using the point at which an abalone can no longer remain attached to the substrate, this being considered the temperature at which no long-term survival is considered likely. Each abalone was weighed, measured by shell length, and tagged with plastic discs, and then placed onto suspended vertical plates (600 mm × 300 mm) in the well-aerated trial tanks. No food was provided for the abalone during the tests. Ambient water temperature was 15 ± 0.2°C for test 1 and 12.5 ± 0.2°C for test 2. The tank was fitted with a 1000 W thermoregulator and the temperature was raised by 2 ± 0.2°C per h until it reached 20°C, after which it was then raised 1 ± 0.2°C per h until none of the animals could keep contact with the vertical plates.

Continual visual monitoring of the trials was undertaken to record any behavioral responses of the abalone during the trials and to record the time and temperature that each abalone detached. Immediately after an abalone had detached, it was weighed and then placed into a well-aerated recovery tank 2 ± 0.5°C lower than the test tank.

Two replicate trials were undertaken for each of the two populations. Different animals were randomly selected for each of the tests. Differences between groups were tested using a 50% CTM calculated by linear regression.

Only bay abalone were used for molecular studies. Before tissue collection, the water temperature was increased by 2°C daily until a temperature of 25°C was reached, representing a total rise of about 9°C. Animals were subsequently removed from the containers and immediately killed by severing of cerebral ganglia. Tissues were quickly removed and snap frozen in liquid nitrogen prior to storage at −80°C.

DNA Extraction

Approximately 0.5 g of frozen tissue from each foot muscle was minced (in a sterile petri dish using scalpel blades) until the tissue formed a coarse paste. Cells were then lysed by shaking the tissue in a 1.5 ml microfuge tube with 1ml of DNAzol (Life Technologies, genomic DNA isolation reagent), and the tube was then centrifuged (13000 × *g*, 10 min, 4°C) (MSE Microcentaur, microfuge). The supernatant was removed and the DNA was prepared by standard chloroform/isoamyl/alcohol precipitation. Extraction was confirmed using separation in agarose gels.

Polymerase Chain Reaction (PCR)

PCR was carried out on genomic DNA in order to gain sequence information from the genes encoding HSP70 in *H. rubra*. PCR on cDNA was used to characterize expressed sequences (mRNA) of HSP70 genes within stressed animals. All reactions were performed using an automated thermocycler (FTS-320 Thermal Sequencer; Corbett Research) and all reactions were layered with one drop of mineral oil prior to cycling.

Primers for PCR were designed from regions of high amino acid (AA) homology seen between aligned sequences of organisms previously cloned. Figure 1 is a general overall positioning of the primers (shown in italics and arrows) on the HSP70 mRNA transcript, together with estimated sizes of PCR products.

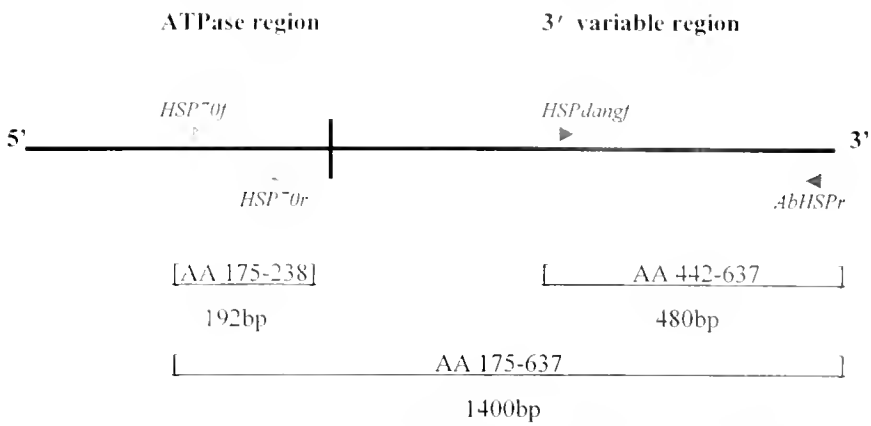

Figure 1. PCR primer binding sites on HSP70 cDNA and expected PCR product sizes.

Genomic DNA (HSP70 192bp): 50 µl reactions containing 200 ng *H. rubra* genomic DNA, 5 µl 10x reaction buffer (Life Technologies), 2–4 mM $MgCl_2$ (Life Technologies), 5 mM of dNTP's (Boehringer), 8 mM of both forward (HSP70f: 5′-GNATHATHAAYGAASCANC-3′) and reverse (HSP70r: 5′-GNATHATHAAYGAASCANC-3′) primers, 2.5 units of *Taq* Polymerase (Life Technologies) and ddH_2O were cycled through 36 cycles (1: 95°C for 5 min; 2–35: 95°C for 120 s, 52°C for 90 s, 72°C for 90 s; 36: 72°C for 5 min).

Genomic DNA (HSP70 1400bp): 50 µl reactions containing 200 ng *H. rubra* genomic DNA, 5 µl 10x reaction buffer (Life Technologies), 2-4 mM $MgCl_2$ (Life Technologies), 5 mM of dNTP's (Boehringer), 8 mM of both forward (HSP70f) and reverse (AbHSPr: 5′-YARTCNACYTCYTCNAC-3′) primers, 2.5 units of *Taq* Polymerase (Life Technologies) and ddH_2O were cycled through 31 cycles (1: 95°C for 5 min; 2–30: 95°C for 120 s, 52°C for 90 s, 72°C for 120 s; 31: 72°C for 5 min).

cDNA (HSP70 192bp): 25 µl reactions containing 1 µl cDNA, 2.5 µl 10x reaction buffer (Sigma), 2-4 mM $MgCl_2$ (Life Technologies), 5 mM of each dNTP's (Boehringer), 8 mM of both forward (HSP70f) and reverse (HSP70r) primers, 1.25 units of RedTaq Polymerase (Sigma) were cycled for 30 cycles (1: 95°C for 5 min; 2–29: 95°C for 90s, 52°C for 90s, 72°C for 60s; 30: 72°C for 5 min).

cDNA (HSP70 480bp): 25 µl reactions containing 1 µl cDNA, 2.5 µl 10x reaction buffer (Sigma), 2-4 mM $MgCl_2$ (Life Technologies), 5 mM of each dNTP's (Boehringer), 8 mM of both forward (HSPdangF: 5′-GAYATHGAYGCNGAYGG-3′) and reverse (AbHSPr) primers, 1.25 units of RedTaq Polymerase (Sigma) and ddH_2O were cycled for 30 cycles (1: 95°C for 5 min; 2–29: 95°C for 90 s, 50°C for 90 s, 72°C for 90 s; 30: 72°C for 5 min).

cDNA (HSP70 1400bp): 25 µl reactions containing 1 µl cDNA, 2.5 µl 10x reaction buffer (Sigma), 2-4 mM $MgCl_2$ (Life Technologies), 5 mM of each dNTP's (Boehringer), 8 mM of both forward (HSP70f) and reverse (AbHSPr) primers, 1.25 units of RedTaq Polymerase (Sigma) and ddH_2O were cycled for 30 cycles (1: 95°C for 5 min; 2–29: 95°C for 90 s, 50°C for 90 s, 72°C for 120 s; 30: 72°C for 5 min).

Purification of PCR Products and Cloning

Fragments amplified by PCR were separated on agarose gels, and, if PCR products were single products, ligations into plasmids were carried out directly from the PCR reaction mixture. Other PCR products were excised from agarose gels under UV light using sterile scalpel blades, and DNA extracted using QIAEX II gel extraction kits (QIAGEN) and QIAquick gel extraction kits (QIAGEN) following the manufacturer's instructions. Alternatively, gel pieces containing desired products, were cut from agarose under UV light and incubated in 50 µl ddH_2O at 60°C overnight, then centrifuged at 13000 × *g* for 15 min to obtain supernatants which were used in reamplification of product via PCR or used directly in ligation reactions.

Ligations of PCR products were carried out within 24 hours of PCR reactions. PCR products were ligated into pCR2.1 plasmid vectors using the Original TA cloning kit (Invitrogen) according to the manufacture's instructions. Plasmids were then transformed into INVαF′ competent *E. coli* cells (Invitrogen) and transformed cells screened by standard blue/white screening procedures. White colonies were chosen as insert-containing clones and screened via PCR directly from colonies using the universal plasmid M13 (forward) and T7 (reverse) primers. Positive clones were selected and a QIAprep spin mini-prep kit (QIAGEN) used to prepare mini-preps of plasmids for subsequent sequencing on an Applied Biosystems Automated Sequencer at Westmead Hospital, Sydney, using the dye terminator method.

RNA Extraction from Frozen Tissue and Production of cDNA

RNA was extracted from foot muscle tissue using a modification of the acid/phenol method (Chomczynski & Sacchi 1987). Frozen tissue (300 mg) was minced finely using sealpel blades and then homogenized with 3ml of TRIzol extraction reagent (Life Technologies), until a cloudy solution was obtained. Samples were centrifuged (13000 × *g* for 15 min at 4°C) in order to remove lipoproteins and fats, and the supernatant removed. RNA was then isolated using the standard chloroform/isopropanol technique, and final pellets washed with sterile 75% EtOH prior to air-drying pellets and storage at −20°C.

First strand cDNA was made from freshly extracted RNA using a Superscript II kit (Life Technologies), according to the manufacturer's instructions. cDNA was synthesised from total RNA and RNase H used to remove the remaining RNA. The cDNA was then stored at −20°C.

Western Blotting

Frozen foot muscle samples (300 mg of tissue) were minced quickly into a coarse paste and the cells lysed by boiling for three

min in 2 ml of lysis buffer (32 mM Tris-HCl pH6.8, 2% SDS, 1 μM PMSF, 2 μg/mL each of pepstatin, chymostatin and leupeptin), followed by homogenization on ice with a teflon pestle (Cole-Parmer, U.S.A.) until all the tissue was suspended. The samples were boiled for a further 5 minutes, followed by centrifugation (13000× *g* for 10 min at RT) and the supernatant collected for protein quantification using a BCA Protein Assay Reagent kit (Pierce), following manufacturer's instructions. Sample concentrations were then equalised by dilution and immediately separated by standard 10% SDS-PAGE, or stored at −20°C until used. Replicate gels were used for electro-transfer of proteins onto nitrocellulose (NC) membranes or Coomassie blue staining.

Dot-blots of Total Protein

NC membranes and two pieces of Whatman chromatography paper were pre-soaked in 1× Western transfer buffer (200 ml MeOH, 5.24 g Tris, 17.6 g Glycine) for 15 minutes. The papers were placed under the NC membrane in a Bio-Dot apparatus (Bio-Rad) and the apparatus assembled. Proteins were prepared in a logarithmic series dilution (10-fold dilutions in lysis buffer) from a 100 μg stock solution (see Western blotting), to 0.01 μg, and boiled for three minutes prior to being loaded and blotted under vacuum. NC membranes were blocked and visualized following probing with specific antibodies as described below.

Immunostaining

NC membranes from Western transfers and dot-blots were blocked with 5% Blotto (5 g skim milk in 100 ml TBS-T), overnight with constant rocking. Membranes were then incubated with a primary (1°) goat anti-human HSP70 polyclonal antibody (Ab) (Santa-Cruz) in a 1:100 dilution with 5% Blotto for 2 h with constant rocking. This Ab was raised against the AA region 572–591 of the C-term of human induced HSP70 and was non-cross reactive with HSC70. NC membranes were washed three times with TBS-T (2.42 g Tris, 5.85 g NaCl, 1 ml Tween-20 made up to 2l with dH_2O), each for 15 minutes before being incubated with a secondary (2°) anti-goat horseradish peroxidase (HRP) conjugated Ab (Life Technologies) in a 1:2000 dilution with 5% Blotto for two h. Membranes were again washed three times each for 15 minutes with TBS-T. Bands were visualized with HRP developing solution (60 mg 1-chloro-4-napthol, 20 ml MeOH, 100 ml TBS, 60 μl 30% H_2O_2) until bands appeared. Results were recorded under white light using an Eagle Eye II still video system (Stratagene). A preabsorption control was performed to confirm that the bands were specific. In brief, 1°C Ab was preabsorbed overnight at 4°C with an excess of the antigen (Santa Cruz).

RESULTS

Responses to Reduced Salinity (Hyposalinity)

All animals were allowed to acclimate in tanks for three days prior to experiments. The shell lengths (mm ± SD) of abalone used in the replicate tests were 115.67 ± 3.2 (test 1) and 111.0 ± 11.47 (test 2) for the ocean abalone, and 108.17 ± 8.4 (test 1) and 111.4 ± 6.02 (test 2) for the bay abalone. Exposure to hyposalinity stress caused swelling of tissue, particularly of the soft tissues behind the head.

Only the data for test 1 are shown in Figure 2, but similar response curves were observed in both tests. Immediately after transfer into the lower salinity medium there was a distinct increase of weight in both abalone groups (ie. approximately 10% at 0.5 h). Following this initial increase the bay abalone decreased their weight over the next 5 h to about that of pre-treatment (ie. 98.8% in test 1; 100.4% in test 2). In contrast, the ocean abalone of both groups did not decrease to the same extent as bay abalone (106.7% in test 1; 102.8% in test 2). The differences between the two groups was significant at 5 h in test 1 ($p = 0.012$) but not in test 2 ($p = 0.083$). Overall, these studies on *H. rubra* populations suggest that the bay abalone are more able to volume regulate.

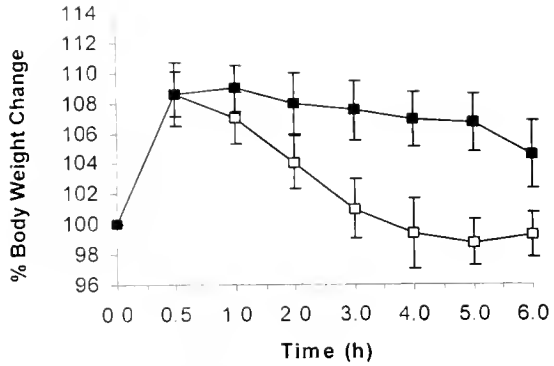

Figure 2. Percent body weight changes of *H. rubra* in response to a hypotonic salinity stress. Abalones from oceanic (■) and estuarine/bay (□) populations were subjected to 80% seawater. Bar lines represent SE of means.

Responses to Elevated Heat Shock

The behavior of the animals subjected to rising temperatures changed at approximately 19°C, when lamellapodia were erected and there was an observed lack of mobility in both ocean and bay abalone. Mucous production was evident in both abalone groups at temperatures above 20–21°C. At about 23°C the abalone raised their shell from their foot by the extension of the adductor muscle and rotated from side to side around its own axis. Above 23.5°C for ocean abalone, and above 24.5°C for bay abalone, retraction of foot from the vertical plates started, and continued until the abalone lost their footing completely. One obvious sign of stress in the more stressed animals was a swelling of the soft tissue around the back of the head, causing it to protrude.

Figure 3 shows the results of test 1 to establish the critical thermal maximum (CTM) for bay and ocean abalone. Replicate tests were performed but there was no significant difference at the $p < 0.05$ level between the groups. Starting from an acclimation temperature of 15 ± 0.2°C, the first ocean abalone in test 1 started to lose hold at 26°C, and bay abalone at 26.7°C, while the last to fall were 28.9°C, and 29°C, respectively. In test 2, starting with an acclimation temperature of 12.5 ± 0.2°C, the first ocean abalone started to drop off the plate at 25.3°C, and bay abalone at 25°C, whilst the last to fall were at 28°C, and 29°C, respectively. The temperature at which 50% remain attached (50% CTM) was calculated at 27.4°C for ocean abalone ($r = 0.92$), and 27.9°C ($r = 0.93$) for bay abalone in test 1, and 26.8°C for ocean abalone ($r = 0.92$), and 27.0°C for bay abalone ($r = 0.95$) in test 2, using linear regression on all points below 100%. The shell lengths (mm ± SD) for the ocean abalone were 116.27 ± 9.41 in test 1 and 115.73 ± 8.80 in test 2, whereas those for the bay abalone were 110.6 ± 7.1 in test 1 and 108.67 ± 4.79 in test 2.

A further test using bay abalone, was performed to obtain tissues from heat-shocked abalone and controls. In this test the water

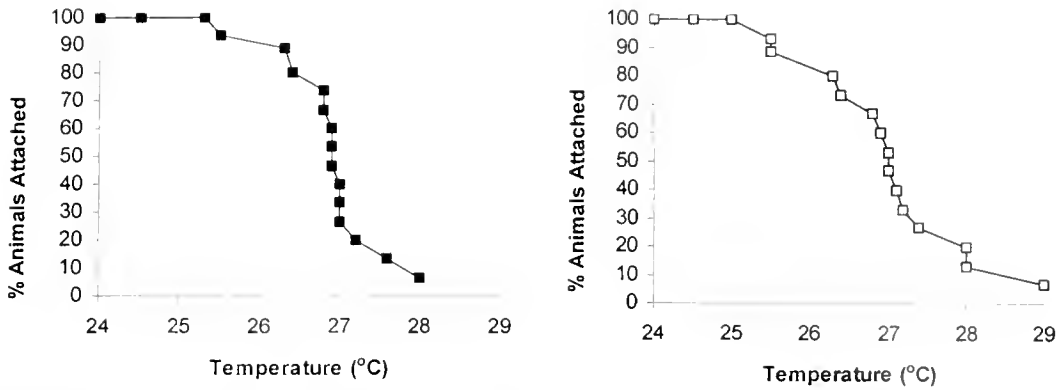

Figure 3. Responses of *H. rubra* to heat stress. Scattergrams showing percent of animals remaining attached as temperature increases for oceanic abalone (■) and estuarine/bay abalone (□).

temperature over the 7-day period for controls was 15.6 ± 2.36° C. The final temperature at which heat stress tissues were sampled was 25.4°C (i.e. an increase of 9.4° C).

Western Blotting

The polyclonal antibody (Ab) raised in goat against human heat inducible HSP70 bound to abalone proteins approximately 70 kDa (Fig. 4). A more intense band was observed in muscle proteins from heat-shocked animals. It is possible that the reactions observed in the salinity stressed or control animals were due to undegraded 70 kDa molecules which had been induced as a result of prior stress, such as transport to the laboratory. In additional Western blots, preabsorption of the Ab with HSP70 protein produced no band, indicating that the HSP70 Ab was specific for abalone HSP70.

Dot-blots of Total Protein

The results of dot-blots on total proteins isolated from heat shocked and control foot muscle of abalone are shown in Fig. 5. There was an increased expression of HSP70 in all 4 heat shocked animals compared with the 4 controls. Heat shocked animals 3 and 4, both of which showed the least stress to heat (ie. attached to substrate and showing no swelling of tissues), also produced the greatest amounts of HSP70, with the detection limit occurring at 1 μg of total protein. This increase in HSP70 was a 10 to 100-fold order of magnitude compared with controls (detection limit 10–100 μg of protein).

Total protein dot-blots were not performed on salinity stress animals, as HSP70 expression in the foot muscle of *H. rubra* did not appear to be upregulated by the stress (Fig. 4).

Cloning of Induced Heat Shock Gene Regions from Abalone Genomic DNA

DNA extraction and purification from *H. rubra* tissues was carried out and confirmed by the presence of a strong DNA band at approximately 23 kb in agarose gels (data not shown). There was almost no shared DNA.

PCR of a 192 bp HSP70 fragment, using HSP70f and HSP70r primers, was not successful. Upon cloning and sequencing of the fragments in three separate attempts, it was apparent 177 bp fragments had been cloned and was not from the HSP70 ATPase region using BLAST searches in Genebank.

PCR of a 1400 bp HSP70 fragment from genomic DNA, using primers HSP70f and AbHSPr, was successful (Fig. 6A). The 1400 bp fragments were cloned and white colonies were obtained with plasmids having the correct size inserts (Fig. 6B). Nested PCR using primers (HSP70f and HSP70r) were used on the plasmid

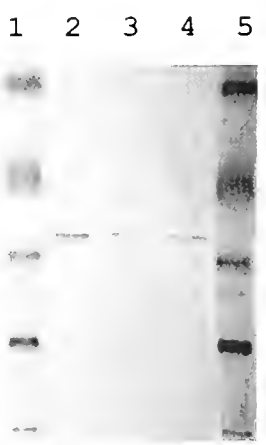

Figure 4. Western blot using a HSP70 specific antibody on total proteins extracted from abalone foot muscles. Western blot showing 70kDa products following membranes being probed with goat anti-human HSP70 polyclonal 1° antibody, then visualised via a HRP conjugated anti-goat 2° antibody. Lanes: 1 and 5, protein MWM of 118, 85, 61, 50 and 38 kDa (Life Technologies); 2, heat shocked abalone proteins; 3, salinity shocked abalone proteins 4, control non-stressed abalone proteins.

Figure 5. Dot-blots of total protein from heat shocked and control *H. rubra*. Membrane showing proteins extracted from foot muscle of heat shocked and control abalone bound and probed with specific goat anti-human HSP70 polyclonal 1° antibody visualised via a HRP conjugated anti-goat 2° antibody. Lanes: 1–4 heat shocked animals; 5–8, control animals. Protein concentrations (top to bottom row): 100 μg, 10 μg, 1 μg, 0.1 μg, 0.01 μg.

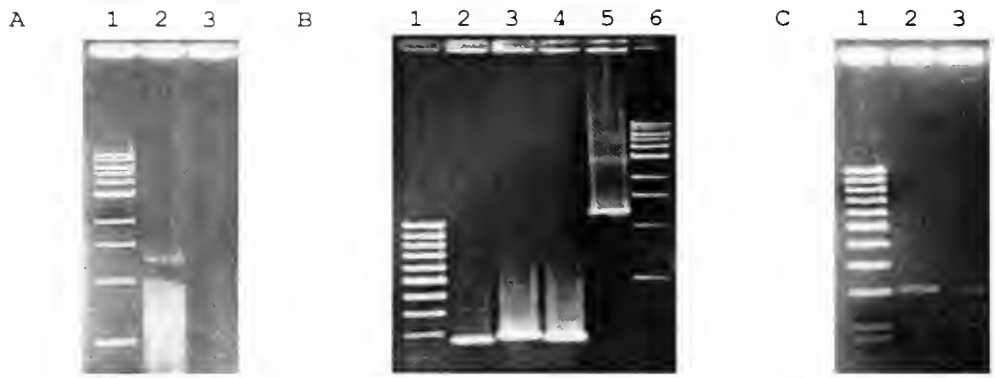

Figure 6. Analysis of PCR products obtained in the cloning of a 1400 bp HSP70 fragment from *H. rubra* DNA using 1.5% agarose gel electrophoresis. A. PCR products amplified using primers AbHSPr and HSP70f. Lanes: 1, 1 kb marker (New England BioLab); 2, 1400 bp fragment plus non-specific bands, 3, negative control (no cDNA). B. PCR screening of bacterial colonies for a 1400 bp HSP70 insert using M13 and T7 vector primers. Lanes: 1, 100 bp MWM (Life Technologies), starting with a 200 bp marker (bottom); 2, negative (no insert, 167 bp of vector); 3 and 4, false positives (small inserts); 5, positive (1400 bp insert plus 167 bp of vector), 6, 1 kb MWM (New England BioLabs). C. Nested PCR on the vector containing the 1400 bp insert using HSP70f and HSP70r primers. Lanes: 1, 100 bp marker (MB1), 2, positive (192 bp fragment); 3, negative plasmid control.

containing the 1400 bp insert, and this gave a 192 bp product (Fig. 6C). These primer sets were subsequently used to identify induced abalone HSP70 sequences in cDNA.

Cloning of Expressed Heat Shock Sequences from cDNA

High quality total RNA (182 ng/μl and a purity of 1.7) was extracted from tissues of abalone that had been heat shocked. This RNA showed characteristic 26s and 18s ribosomal RNA (rRNA) bands amidst other RNA's. The conversion of mRNA into cDNA was confirmed by successfully performing PCR on the cDNA using actin specific primers to produce a 474 bp fragment with >84% homology to three invertebrates (data not shown).

PCR of a HSP70 192 bp fragment from heat shocked abalone foot muscle cDNA gave a product running at the appropriate size (Figure 7A). This fragment was purified and cloned to produce white colonies containing plasmids with the inserts (Fig. 7B). Sequencing of a plasmid insert, along with similarity searches in GeneBank using BLAST, revealed that the clones were part of an abalone HSP70 sequence (Fig. 8). The sequence of 192 bp aligned with the same nucleotide sequence of other organisms with most homology to *B. glabrata* and *Drosophila* at 76% and 73%, respectively (Fig. 8A). A deduced amino acid sequence (AA), when aligned with known AA sequences, showed highest homologies to two other molluscs, *B. glabrata* and *A. californica*, exhibiting homologies of 87% and 84%, respectively (Fig. 8B).

The 192 bp sequence was also compared with known constitutive heat shock proteins (HSC's) and relatively lower homologies were observed in this region of *H. rubra* HSP70 (Fig. 8C). As well, these HSC70 sequences all had the GTC triplet (see Fig. 8A), encoding valine, absent. These data give a strong indication that the sequence obtained is in fact that from an induced HSP70.

PCR of a 1400 bp and 480 bp fragments from abalone heat shock cDNA were successful. However, several attempts to re-amplify and clone these fragments were unsuccessful.

DISCUSSION

The blacklip abalone (*H. rubra*) is primarily found in southern Australian waters and is an economically important species to Australia, worth millions of dollars a year through exports alone. The condition of the animals in wild-catch and cultured stocks, including meat weight, can affect their economic value. Stress is especially seen in summer and during periods of high rainfall which affect water temperature and salinity. These conditions apply particularly to those species in estuarine/bay environments. The heat shock response (HSR) is imperative to the survival of organisms and protects against protein aggregation and cellular damage, notably when an organism is under stressful conditions. These stressful conditions lead to the upregulation of HSPs, including HSP70.

Our research indicates that the bay abalone are more physiologically adapted to respond to hypo-osmotic stress than the ocean abalone. Most marine molluscs, including presumably abalone, are osmoconformers, which means that the extracellular fluid is almost iso-osmotic with the environment. Usually, such animals

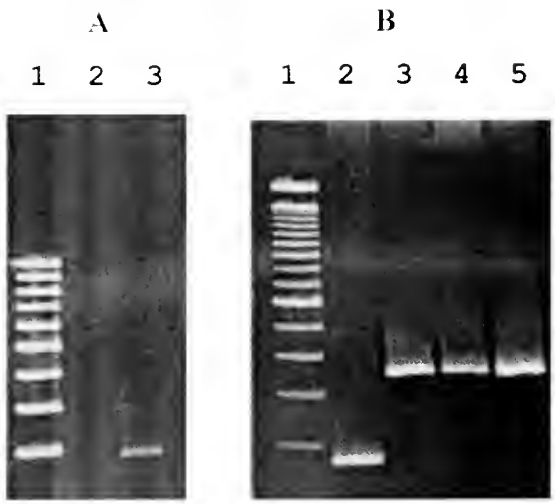

Figure 7. Analysis of PCR products obtained in the cloning of a 192 bp HSP70 fragment amplified from *H. rubra* cDNA using 2.0% agarose gel electrophoresis. A. PCR using HSP70f and HSP70r primers. Lanes: 1, 100 bp ladder (MB1), starting with a 200 bp marker (bottom); 2, DNA negative control; 3, 192 bp product, which was subsequently isolated for TA cloning. B. PCR screening of white colonies for a 192 bp insert using primers M13 and T7. Lanes: 1, 100 bp ladder (MB1); 2, negative (167 bp of vector pCR2.1 with no insert); 3, 4 and 5, positive (359 bp including a 192 bp insert).

A

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H. rubra          (  1)   GGATTATCAACGAGCCGACGGAAGCTGCACTGGCGTATGGCTTGGACAAG
B. glabrata       (  1)   .A..A.....T.....C..A.CC.....C.....A......C.T.....A
D. melanogaster   (  1)   ....C..........C....CG..G........C..C..GC........

H. rubra          ( 51)   AATCTGAAAGGTGAGAAGAATGTATTGGTGTATGATCTGGGAGGCGGTAC
B. glabrata       ( 51)   GG..AT.....A..........T...A.T.TC..C.....T..T..C..
D. melanogaster   ( 51)   ..C..CC.G..G...CGC..C..GC.CA.C.TC..CT....C.....C..

H. rubra          (101)   CTTCGACGTCTCCGTCCTCACCATCGACGAAGGGTCGATGTTTGAAGTGA
B. glabrata       (101)   T.........AG.A..T.G........T...................C.
D. melanogaster   (101)   ............A....G..........G..A..TC....C..G..CC

H. rubra          (151)   GATCAACAGCAGGGGATACCCATCTCGGAGGCGAGGACTTCG
B. glabrata       (151)   AGG.T..T..T..C..C........G.....T..A.....T.
D. melanogaster   (151)   .GG.C..C..C..A..C..GG.C..G..C..T..........
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B

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H. rubra          (  1)   IINEPTEAALAYGLDKNLKGEKNVLVYDLGGGTFDVSVLTIDEGSMFEVR
B. glabrata       (  1)   ......A.........GH.......IF..........I...........K
A. californica    (  1)   ......A.........GQ....H..IF............A.....I...K

H. rubra          (51)    STAGDTHLGGEDF
B. glabrata       (51)    A............
A. californica    (51)    A............
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C

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H. rubra(HSP)     (  1)   ATTATCAACGAGCCGACGGAAGCTGCACTGGCGTATGGCTTGGACAAGAA
A. californica(HSC)(  1)  ..C.....T..A..C..A.CC.....CA.T..C..C..TC..........
C. griseus (HSC)  (  1)   ........T..A..A..T.CT.....TA.T..T.....GC.A..T.....
D. rerio (HSC)    (  1)   ..C.....T..A..A..T.CT.....TA.T..T.....T........A..

H. rubra(HSP)     ( 51)   TCTGAAAGGTGAGAAGAATGTATTGGTGTATGATCTGGGAGGCGGTACCT
A. californica(HSC)( 51)  AG.TGGCAC.....G...C..TC.CA.C.T......T..T..T.......
C. griseus (HSC)  ( 51)   GG.TGG..C...A.G......GC.CA.T.T...CT.......T.....T.
D. rerio (HSC)    ( 51)   GG.TGGT.C.....GA.....CC..A.T.T......T..T..T..C..T.

H. rubra(HSP)     (101)   TCGACGTCTCCGTCCTCACCATCGACGAAGGGTCGATGTTTGAAGTGAGA
A. californica(HSC)(101)  ....T..G...A..........T..G..C..---C..C.....G....A.
C. griseus (HSC)  (101)   .T..T..G..TA.......T..T..G..T..---A..T........C.A.
D. rerio (HSC)    (101)   .T..T..G..AA.T........T..G..T..---C..C..C..G..C.A.
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Figure 8. Sequence alignments of HSP70 ATPase region from a 192 bp PCR fragment of *H. rubra* heat shock cDNA compared with other invertebrates. A. Nucleotide sequence shows homologies of 76% and 73% with others HSP70s. Letters in bold print represent primer sequences. B. Deduced amino acid sequence shows homologies of 87% and 84%, respectively (AA residues 175–238 of HSP70s). C. HSP70 nucleotide sequence shows homologies of 69%, 69% and 68% with HSC70s. Region in italics and underlined indicates a region of low homology. Three nucleotides in bold (encoding valine) are absent in HSC70s.

protect cells from volume perturbation, due to altered salinity, by modulating the concentration of free amino acids and other organic solutes in the intracellular compartment (Burton 1983). In these marine invertebrates exposed to low salinity, a rapid increase in fluid volume is compensated in a matter of hours (good volume regulators) or days (weak volume regulators) (Oglesby 1981, Davenport 1985). The success with which an osmoconforming species tolerates environmental dilution can vary between populations of the same species, depending on the variability of the salinity normally encountered in the environment. For example, in a study on the bivalve, *Geukensia demissa,* animals from an estuarine environment tolerated reduced salinities better than conspecifics from a higher salinity site (Garthwaite 1989). In the current study, we observed that the Port Phillip Bay population, which is exposed to a greater salinity range than oceanic populations, compensated for volume gain in reduced salinity faster and more effectively than the oceanic abalone (Fig. 1). Such a difference may be genetically determined because the bay abalone have a greater than 12% genetic difference compared to the oceanic populations (Huang et al. 2000). Studies on other molluscs have indicated that genetic variation at the *LAP* locus is correlated with populations which are distributed along a salinity cline (see for example, Garthwaite 1989, Gardner & Palmer 1998). However, we were not able to correlate environmental salinity with changes in the expression of HSP 70.

In our heat stress experiments, the upper lethal temperature for *H. rubra* was not determined, but the 50% CTM and the final temperature at which all animals had dropped off the plates were not significantly different. Similar 50% CTM's have been observed for *H. midae* at 27.9°C (Hecht 1994), and *H. rubra* at 26.9 C

and *H. laevigata* at 27.5°C (Gilroy & Edwards 1998). Hecht (1994) infers that differences in 50% CTM are considered to be of a greater zoogeographic significance than fixed physiological differences since they reflect adaptation to local environmental conditions. We had expected differences in 50% CTM as the bay populations can experience summer seawater temperatures of 30°C for short periods (unpublished), and there appears to be approximately a 12% genetic divergence between bay and oceanic populations (Huang et al. 2000).

In the current study, attempts to characterise the HSR in *H. rubra* during stress by expression of HSP70 proteins, proved to be successful. Proteins were identified from the foot muscle of abalone through Western blotting and upregulation of HSP70 was observed in heat-stressed animals via dot-blots of total protein. All sample groups in the experiments showed HSP70 expression with heat-shocked animals displaying obvious upregulation over salinity-stressed and control animals. Control and salinity-stressed animals did display some expression of the HSP70 protein. Given that antibodies were specific for an induced HSP70, it may be assumed that control animals in these experiments were under some form of minimal stress and were expressing induced HSP70, which may be basal level expression. All animals would also be expressing HSC70s, the non-induced form of the HSP70 family, but these proteins could not be monitored because of specific recognition of the antibody for the induced member. This assumed basal expression of HSP70 might not necessarily be basal expression at all, but a response to one of many stresses the control animals experienced during their transport and maintenance, similar to that observed in a study of the HSR response in *Mytilus californianus* (Roberts et al. 1997). Therefore, it would be favorable to execute the experiments after one or two months of moving the animals to the new environment because, after initial expression, HSP70s are not degraded for approximately two weeks, assuming no further stress is encountered (Sanders 1993).

Upregulation of HSP70 during heat shock, as seen by the 100-fold increase in expression of HSP70 proteins in *H. rubra*, has been previously well-documented in a variety of organisms, including other marine animals. A reported maximum 1000-fold increase of HSP70 has been observed after a heat shock in *D. melanogaster* (Parsell & Lindquist 1994), but levels resembling those of *H. rubra* have been monitored in oyster haemocytes (Tirard et al. 1995), cells originating from the fresh water snail *B. glabrata* (Laursen et al. 1997), the mussel *M. californianus* (Roberts et al. 1997), and the lamprey species *Petromyzon marinus* and *Lampetra appendix* (Wood et al. 1998). Genetic differences in the HSR between closely related species was reported in *Collisella* limpets, *C. scabra* and *C. pelta* (Sanders et al. 1991). This study monitored HSP expression under heat stress in both species, and found HSP70 expression was induced at lower temperatures, and at increased levels, in *C. scabra*, compared with *C. pelta*.

An upregulation of HSP70 was not observed in salinity-stressed *H. rubra*. This lack of increased expression of HSP70 does not imply that the HSR was not engaged in *H. rubra*, as it could be due to the expression of proteins other than HSP70. This has been observed in *Eurytemora affinis* (Gonzalez & Bradley 1994). In this study, osmotic shock did not increase the expression in HSP70. However, new proteins in the ranges of 24–29 kDa, 45–50 kDa, 55–65 kDa and 75–85 kDa were expressed in response to changing salinities. Because the antibody used in our *H. rubra* study was against HSP70 specifically, it would not detect other types of expressed proteins. In contrast, HSP70 was induced by hyperosmotic salinity stress in *Salmo salar* (Atlantic salmon), together with another stress protein, Osp54 (Smith et al. 1999). The increase in expression of proteins was accompanied by an overall decrease in other cellular proteins during salinity stress, which suggests that stress proteins were preferentially expressed. These findings present the first substantial evidence that HSP70, specifically, is expressed under salinity stress and it is hypothesised to function in restoring osmotic homeostasis and renaturing proteins that have been destabilized during osmotic stress.

The molecular cloning of HSP70 fragments using PCR of genomic DNA and cDNA was initiated to characterise and identify genes of *H. rubra* that may be induced in response to stress. In particular, sequencing of the 3′ variable end of an expressed mRNA would be useful in production of oligonucleotide probes for studying regulation of HSP70 in northern blots and for cDNA library screening.

The cDNA sequence of the 192 bp region of HSP70 ATPase region had high homology to HSP70s of other species. This sequence was unlikely to be constitutive HSC70 because there was less homology (<69%). In addition, a triplet at position 218 was absent from all the HSC70s. The HSC70s contained a region of low homology (encoding AA positions 188–191 in HSP70). It has been reported that position 190 in the HSP70 AA chain is a region not involved in tertiary structure formation in the overall functional HSP70 protein, and is therefore prone to mutation (McKay et al. 1994). The overall evidence indicates that the sequence obtained from *H. rubra* was in fact that from the heat-induced HSP70.

The two larger fragments PCR amplified from cDNA (viz 1400 bp and 480 bp) show that we now have tools to further investigate the nature of the HSR in abalone. Sequence data, particularly of the 3′ end of an induced HSP70, would provide probes for future use in expression patterns of HSP70 mRNA and HSP70 proteins during stress. Such techniques have been employed previously using HSP70, where a nucleic acid HSP70-binding-protein was detected (Raynes & Guerriero, 1998).

Overall, the current study has shown that abalone respond to heat stress with expression of HSP70. The identification of additional molecules induced by heat stress, and the identification of a constitutive HSC70, are still to be carried out. In addition, the physiological factors responsible for the difference in volume regulation ability between bay and oceanic abalone remain to be elucidated.

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LOCATION OF EGG-LAYING HORMONE IN REPRODUCTIVE STRUCTURES AND NEURONS OF *HALIOTIS RUBRA* (LEACH) USING ANTIBODIES RAISED AGAINST RECOMBINANT FUSION PROTEINS

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ABSTRACT Recombinant abalone egg-laying hormone was produced using a bacterial expression vector. This required directional cloning of a 108-bp abalone egg-laying hormone (aELH) gene sequence, using PCR of genomic DNA with primers incorporating restriction enzyme sites, into a pGEX-2T vector. Following transformation of the recombinant vector into *Escherichia coli*, a GST:aELH fusion protein was produced in large quantities and then purified. This fusion peptide was used to immunize mice for the production of polyclonal and monoclonal antibodies, which were subsequently tested for specificity using ELISA's and Western blots. Antisera and two IgM monoclonal antibodies were shown to react with aELH. These antibodies were used in immunocytochemistry studies of neural and gonad tissues of sexually mature female abalone. The aELH was found to be located in neurosecretory cells of cerebral and pleuro-pedal ganglia, statocysts, and trabeculae in female gonads.

KEY WORDS: egg-laying hormone, aELH, fusion protein, monoclonal antibodies, immunocytochemistry

INTRODUCTION

Immunocytochemistry has previously been used to characterize central neurons which react to antibodies raised against neuropeptides, including the α-caudodorsal cell peptide (α-CDCP) and caudodorsal cell hormone (CDCH) in *Lymnaea stagnalis*, and corresponding egg-laying hormone (ELH) in *Aplysia californica* (Van Minnen et al. 1989, Nambu & Scheller 1986). These antibodies allowed the identification of the neurosecretory cells controlling egg laying. In *Lymnaea*, the neurosecretory cells are located within the caudo dorsal cells (CDC) of the cerebral ganglia. The CDCs are arranged in two clusters in the left and right cerebral ganglia. In *Aplysia*, bag cells of the abdominal ganglion appear immunoreactive for ELH. The bag cells consist of two clusters of 250 to 400 cells located in the abdominal ganglion, near the origin of the pleurovisceral connectives.

Further investigations have indicated that the egg-laying preprohormone is relatively conserved across a wide range of molluscan classes (Nambu & Scheller 1986), resulting in the identification of additional peptides associated with neurons, which possibly control egg-laying and egg-laying behavior. Van Minnen et al (1992) have found that a number of gastropod molluscs, including *Helix aspersa*, *Biomphalaria glabrata* and *Limax maximus*, contain immunoreactive proteins using antibodies raised against α-CDCP, a short peptide within the CDCH preprohormone. Using these antibodies, as well as antibodies against CDCH in cross-reaction studies, it has also been shown that neurons in the bivalves *Mytilus*, *Mya* and *Placopecten* also contain a similar vitellogenic factor (Croll et al. 1993). These selective immunological markers, therefore, suggest that related peptides may be involved in the egg laying of a wide range of gastropod and bivalve molluscs.

In addition, peptides immunoreactive to antisera specifically directed against CDCH, α-CDCH and β-CDCH (another short peptide in the CDCH preprohormone), have been detected in the central nervous system of the rhyonobdellid leech, *Theromyzon tessulatum* (Salzet et al. 1997). As well, it has been shown that polyclonal antisera directed against the same three peptides results in positive immunoreaction in *Sarcophaga bullata* (Diptera), *Leptinotarsa decemlineata* (Coleoptera), *Locusta migratoria* and *Periplaneta americana* (Orthoptera) (Theunis et al. 1990).

Abalones are classified as primitive gastropods and have a nervous system containing cerebral ganglia rather than a defined brain. Nerve cords and connectives lead from the cerebral ganglia, two of which join to a single pleuro-pedal ganglion. This ganglion is composed of fused pleural and pedal ganglia, both of which are elongated and flattened (Crofts 1929). A common feature of all these ganglia is the possession of an outer cortex of ganglion neurons and glial cells surrounding a central neuropil. The small neuronal cells and glial cells lie below the surface layer but do not extend into the neuropil.

Neurosecretion in the cerebral ganglia was investigated in *H. discus hannai*, in order to determine the role of hormones in the regulation of reproduction (Hahn 1994). The study showed that four types of cells exist in the cerebral ganglia, and these were defined as cell types A, B, C and D, of which only cell types A and B appear to be neurosecretory. Cell type A shows a correlation with vitellogenesis and gametogenesis in the ovary (Hahn 1994). More recently, similar neurosecretory cells of cerebral ganglia have been identified in *H. asinina* (Upatham et al. 1998). In other experiments, Yahata (1973) induced spawning of abalone by injection of homogenized pleuro-pedal and visceral ganglia of mature females, indicating the existence of an egg-laying hormone. However, injections of homogenized cerebral ganglia produced no notable change in the ovaries. Despite these cytological and physiological studies indicating the existence of sets of neurosecretory cells in the ganglia, there is no definitive evidence that peptides produced in these cells are regulating reproduction and growth.

The nucleotide sequence of an abalone ELH (aELH) has been recently obtained for *H. rubra* (Wang & Hanna 1998), and other abalone sequences (Hanna et al. 2000), all of which show high homology. Fig. 1 shows the egg-laying precursor peptide arrangements of *L. stagnalis* and *A. californica*, together with the location of the sequence encoding the aELH of *H. rubra*. The nucleotide homology of the aELH is 94% compared with the CDCH of *L. stagnalis* and 56% compared with the ELH of *A. californica*.

Despite this, very little is known about the regulation of egg-laying hormones during reproductive cycles in the genus *Haliotis*, and the sites at which aELH is expressed. Therefore, research was

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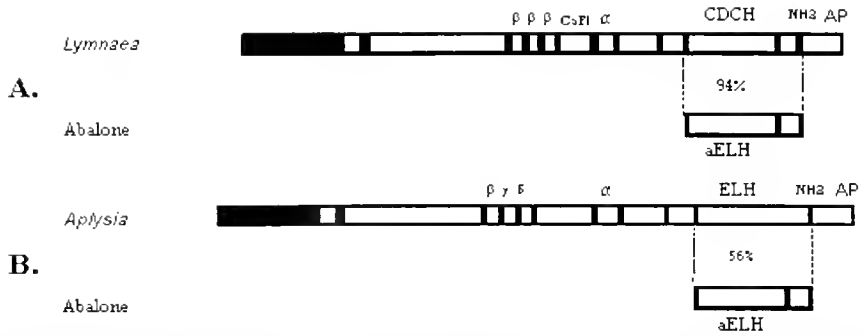

Figure 1. Comparison of *Lymnaea* CDCH (A, Vreugdenhil et al. 1988) and *Aplysia* (B, Scheller et al. 1982) egg-laying preprohormone sequences with the known abalone sequence. NH2 represents a potential amidation signal. Percentage nucleotide homology between corresponding regions are indicated. Positions of potential peptides are indicated by β_{1-3}-CDCP/β-BCP, γ-BCP, α-CDCP/αBCP, δ-BCP, calfluxin (CaFl) and an acidic peptide (AP). Known or potential cleavage sites are shown with black bars, and the larger black regions (left) represents signal sequences at the 5′ region.

carried out to develop antibodies for subsequent use in immunological techniques to locate cells producing egg-laying hormone within abalone neural and reproductive organs.

MATERIALS AND METHODS

Recombinant Protein Expression and Purification

Recombinant aELH was expressed in large quantities and purified by the use of a pGEX-2T (Amersham) expression vector in which the 108-bp egg-laying hormone sequence of *H rubra* had been inserted (Wang & Hanna 1998). This recombinant plasmid was transformed into *E. coli* competent cells and colonies selected on LB medium containing ampicillin. A 10-mL bottle of prewarmed 2× LB/Amp medium was loop inoculated with cells from one colony, and incubated at 37°C with shaking overnight. This culture was transferred into 1 L of the same medium and grown at 37°C with shaking until A_{600} reached 0.5-0.2. Bacterial fusion proteins were induced by adding 0.5 mL of 100mM IPTG with incubation for another three to five hours. The culture was centrifuged at 8,000 rpm for 5 min at 4°C (Beckman, JA-20) in 40-mL conical flasks. The supernatants were discarded and the tubes placed on ice. The pellets were resuspended in ice-cold PBS and the cells sonicated for five 30-second intervals on ice, ensuring that no frothing occurred. Immediately following this, 10% Triton X-100 was added to make final concentrations of 1% and the solutions were then incubated for 30 minutes with gentle mixing on ice to aid in solubilization of fusion protein. The suspensions were centrifuged at 10,000 rpm for 10 min at 4°C and supernatants transferred to 50-mL centrifuge tubes. Glutathione sepharose 4B beads were mixed with each 100 mL of sonicate and the contents left to stand for one hour. The glutathione sepharose 4B beads with fusion protein were sedimented by centrifugation and the supernatant collected for later reference. Ice-cold PBS was added and beads washed three times by repeated centrifugations. The fusion protein was then eluted with glutathione elution buffer, and analysis of fusion protein was performed by SDS-PAGE and Coomassie blue staining.

Production of Antibodies

Antigens used in primary immunizations were prepared by mixing 50 μg of the fusion protein with an equal volume of Freund's complete adjuvant (Sigma). BALB/c mice were then immunized intraperitoneally. Subsequent immunizations were performed each wk, for 3 wks, using the same dose of fusion protein but mixed with Freund's incomplete adjuvant (Sigma).

Polyclonal antisera were obtained by tail-bleeding mice and stored for use when ELISA end-point titres of >10,000 were obtained. For immunocytochemical studies, antisera were diluted 1/500 to 1/5000. Monoclonal antibodies were prepared four days after the last injection of immune mice, using standard techniques (Goding 1987). The mice were killed by cervical dislocation and the spleens removed aseptically. Spleen cells were fused with Sp2/0-Ag-14 myeloma cells using PEG 4000. The supernatants of the hybridomas surviving the selective medium consisting of RPMI 1640, bovine fetal calf serum and HAT, were tested for the presence of specific antibodies by means of ELISA. Antibodies secreted from positive clones were isotyped and the cells re-cloned twice to ensure monoclonality.

Quantification and Specificity of Antibodies in ELISA

ELISAs were undertaken to determine the titre of anti-aELH sera obtained from mice and for screening hybridomas and specificity of reaction with antigen. Antigen concentrations, which were calibrated to 20 μg/ml, consisted of expressed GST-aELH fusion protein or expressed GST.

The wells of a microtitre plate (Bio-Services) were coated with 50 μL of antigen solution, which was allowed to adsorb to the plate at 4°C overnight. Unoccupied binding sites on the plates were blocked with 1% BSA in coating buffer for one hour at 37°C. The plates were then washed three times in washing buffer and incubated with 50 μL of polyclonal antiserum diluted to 1:50, 1:100, 1:500, 1:1000, 1:5000 and 1:10000, in incubation buffer at 37°C for one hour. Supernatants of hybridomas were substituted for the antisera in additional tests. For the detection of bound antibody, plates were washed three times in washing buffer and incubated for one hour at 37°C with 50 μL per well of a secondary alkaline phosphatase labeled antibody (Sigma) diluted 1000-fold in incubation buffer. Plates were then washed three times in washing buffer and reacted with a 50 μg/mL solution of the enzyme substrate. After 100 min of color development, the plates were read on an automated ELISA plate reader (Titertek Multiskan MCC/340) at 405 nm.

Dissection of Abalone Tissues

Live female *H. rubra* (Leach) with mature gonads were collected under a research permit (97/R/049A), during the months

September to November. If not used immediately they were transferred to an abalone aquaculture facility where they were kept for no more than 5 days under a 12-h light/12-h dark cycle. Animals were removed from the shell prior to dissection.

To remove the left and right cerebral ganglia (cg), a blunt probe was used to separate the tissues from each side of the head. The cg with two nerves protruding was then detached. Next, the pleuropedal ganglia (ppg) and statocysts were exposed by removing all organs in the head cavity with a scalpel and then removed from within the muscle tissue. A piece of female gonad was also removed from the conical end of the organ.

Fixation and Sectioning of Tissues

After dissecting out the ganglia and pieces of gonad, they were fixed in freshly prepared 4% paraformaldehyde at 4°C for 24 h. They were then transferred to phosphate buffer, and stored at 4°C before further processing.

Prior to cryosectioning, tissues were transferred to 30% sucrose in phosphate buffer and stored overnight at 4°C. The preparations were then frozen in TissueTek (Bayer Diagnostics) in the cryostat at −20°C and subsequently sectioned at 6 μm. Alternate sections were placed on separate slides so that different antibodies or control procedures could be compared. Slides were then stored at −80°C, until required.

Western Blots

Standard SDS-PAGE of expressed GST and GST-aELH were carried out prior to Western transfer of the proteins onto 0.45 μm nitrocellulose membranes. The membranes were then reacted with antisera or mAbs to determine the sizes of antigenic proteins.

Fresh tissues of cg, ppg and gonad were used to prepare total protein extracts using TriPure isolation reagent (Roche), according to the manufacturer's instructions. These extracts were separated on SDS-PAGE. Proteins were transferred, as before, and probed with antisera or mAbs.

Immunocytoehemistry

A DAKO LSAB2 (streptavidin-biotin labeled) kit was utilized to identify the location of aELH in sections. Initially, sections were heated in buffered citrate (pH 6.0) to enable antigen retrieval. To prevent endogenous peroxidase activity, the slides were treated with 3% hydrogen peroxide, after which, primary antibody (diluted polyclonal or monoclonal in ascites fluid) was added and the slides incubated for 1 h at RT. After washing with PBS, biotinylated anti-mouse immunoglobulins were added and the slides incubated for 30 min at RT. After a brief washing, two drops of streptavidin peroxidase were added and another incubation carried out at RT for 20 min. During this step, a fresh solution of 3-amino-9-ethylcarbazole (AEC) chromogen was prepared. The chromogen was added after another washing and a further incubation at RT for 3 to 5 min performed, before being examined for color development. When appropriate, color development was stopped by washing the slides in distilled water, a counterstain in Mayer's haematoxylin performed, and the slides washed again and before mounting in Faramount aqueous mounting solution (DAKO). Digital images were then taken using a Zeiss Axioskop MC 80.

Immunofluorescence was performed on stored frozen sections. The sections were allowed to thaw at RT for 30 min before blocking, first in 1% glycine for 30 min, then in 4% BSA for 30 min. They were rinsed three times in PBS, and then incubated for 1 h at room temperature in primary antibody (i.e. polyclonal or monoclonal). After three rinses in PBS, they were incubated in FITC-labeled goat anti-mouse secondary antibody (Silenus) in the dark at RT for 1 h, and then rinsed three more times in PBS, before mounting in FITC mounting solution. Sections were viewed under blue light with a Zeiss Axioskop MC 80 microscope and images recorded.

Negative control procedures included the omission of primary antibodies and preadsorbing the anti-aELH antibody with recombinant aELH.

RESULTS

Monoclonal Antibodies

Table 1 shows the properties of five monoclonal antibodies (mAbs) that were produced with medium to strong reactions in ELISA and immunofluorescence testing. MAbs with weak reactions were discarded. Three mAbs (i.e. F61P3E3, F62P1A1 and F62P6D4) reacted with the GST component of expressed GST-aELH fusion protein as well as expressed GST. The other two mAbs (i.e. F61P1A5 and F62P4C1) reacted with the aELH component of expressed GST-aELH fusion protein. It was unusual that most of the mAbs were IgM as a period of four days from the last immunization of peptide to the production of mAbs would have allowed for class-switching of cells from IgM to IgG's.

Specificities of mAbs were also confirmed using Western blots of expressed GST and GST-aELH fusion proteins (data not shown).

Western Blots of aELH in Abalone Tissues

The results of Western blots of cg, ppg and mature female gonad proteins are shown in Fig. 2. There were no bands at approximately 4 kDa, the estimated size of aELH (36 amino acid residues deduced from a 108 nucleotide sequence), in any of the four experimental lanes. This absence may be attributed to the small size of the aELH (i.e. 4 kDa) and relatively small amounts present, compared with the total protein present. However, the cg showed 6 bands of sizes ranging from 24 to >77 kDa, the ppg 3 bands of which two were between 34 and 48 kDa and the other >77 kDa, and the gonad showed two bands between 34 and 40 kDa. The presence of these multi-bands indicates the different processing of preprohormones in the tissues and/or the presence of multigene products.

TABLE 1.

Monoclonal antibodies produced and their properties

Monoclonal Antibody	Isotype	Specificity in ELISA	Reactivity using Immunofluorescence
F61P3E3	IgM	GST	+++
F62P1A1	IgM	GST	++
F62P6D4	IgG_1	GST	+++
F61P1A5	IgM	aELH	++
F62P4C1	IgM	aELH	++

+++, strong reaction; ++, medium reaction; +, weak reaction (mAb discarded)

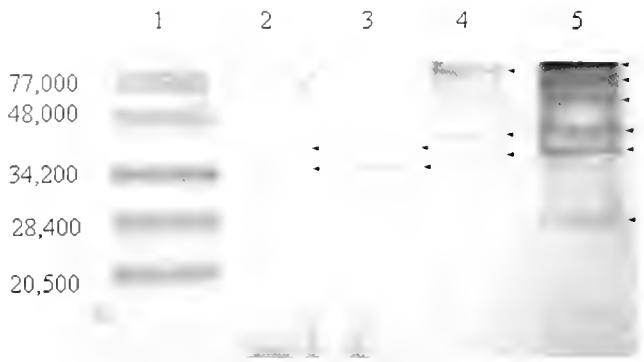

Figure 2. Western blots of proteins from *H. rubra* tissues using anti-aELH antibodies. Lanes: 1, pre-stained molecular weight markers (Bio-Rad); 2 & 3 mature female gonad; 4, pleuro-pedal ganglia; 5, cerebral ganglia.

Immunocytochemistry

A brick-red precipitate of the immunological test, together with a blue haemotoxylin counter stain, gave clear indications of antigen location. We had initially used 3,3′ diamino-benzidine tetrahydrochloride (DAB) in the final reaction, to give a brown precipitate, but found that there were endogenous brown pigments in control tissues (data not shown). This made it difficult to determine the location of the immune reaction, so this method was substituted by the AEC substrate to give a clearer result.

Consequently, immunopositive reactions were observed in eg, ppg, and mature female gonads (Fig. 3). However, there were marked differences in each of the structures. The cells reacting in the eg were equivalent to the NS1 neurosecretory cells observed by Upatham et al. (1998). In the ppg, peripheral neurosecretory cells have also been identified by the same group (unpublished), and our current work showed these cells immunoreactive. Immunoreactive material was also observed around the statocysts. In the gonads, a small number of cells spread throughout the trabeculae showed strong immunoreactions.

The results obtained using immunofluorescence location of aELH were the same as for immunoenzyme staining (Fig. 3).

DISCUSSION

Production of immunocytochemical probes has relied extensively on the use of native protein to provide specific polyclonal and monoclonal antibodies. However, there has been a transgression toward the use of synthetic or recombinantly expressed proteins, to facilitate antibody probe production. The advantage of these proteins is three-fold: large amounts of protein can be produced; only partial sequence data needs to be obtained; and laborious protein purification techniques can be avoided. In this study, the aELH sequence data obtained (Wang & Hanna 1998) was used for the *in vitro* expression and subsequent immunization of experimental animals. Antibodies were then produced that with immunoreactivity toward the recombinant protein in ELISA. However, further analyses were required to determine immunoreactivity to native protein *in vivo*, and this was ratified by immunocytochemical studies. This was not unexpected as previous studies have shown that antibodies raised against native ELH protein of *Aplysia* is immunoreactive against a synthetic ELH peptide.

In addition to ELISA, further specificity of the monoclonal and polyclonal antibody immunoreactivity to aELH was achieved by Western blots. These confirmed that the antibody probes were reactive to a protein extracts from the central nervous system and the gonad. The antibodies against aELH recognized proteins of various sizes within gonad, cerebral and pleuro-pedal ganglia extracts. This indicates that there are a number of polypeptides of different sizes, each containing the aELH peptide component. These bands probably represent differential cell processing of the polypeptides, typical of that found in *A. californica* (Fisher et al. 1988). They showed by Western blot analysis that antibodies recognized small final-product peptides, intermediates in the processing pathway which contain the sequence used as the immunogen, and also the large prohormone. However, the multi-band data may also be the result of the presence of a multigene aELH family, the genes of which are expressed in different tissues. We have preliminary data (unpublished) indicating that abalone have several genes encoding aELH. Our data is consistent with the findings of Nambu and Scheller (1986), in their studies of *Alysia* genomes. They used Southern blotting, gene cloning and immunocytochemical techniques to identify and characterize ELH related genes. Overall, there is a need for a substantial investigation into the number of aELH related genes in the abalone genome, the cellular expression of these genes, and the processing of preprohormones in each tissue to give immunoreactive proteins of various sizes.

In vivo expression of the female *H. rubra* aELH was shown in our studies of sections of abalone tissues, using monoclonal and polyclonal antibodies. The aELH appeared to be localized to the NS1 cells within the cerebral, and were also found in pleuro-pedal ganglia, statocysts, and to the trabeculae of the gonad. Our data supports the previous research by Hahn (1994) and Upatham et al. (1998), in which they showed that differential staining could identify neurosecretory cell types in the cerebral ganglia. In addition, the cerebral ganglia of other molluscan species are known to be a major site of ELH-like expression (Croll et al. 1993, Van Minnen et al. 1992).

Immunoreactivity within the pleuro-pedal ganglia was not only localized to the cells of the periphery, but also the statocyst margins and interior. The statocyst is a chambered sense organ containing granules to sense the direction of gravity.

Despite extensive research being conducted on neuropeptide immunoreactivity within the central nervous system, little research has focused on the reproductive tissue. Our study, however, shows aELH located in the trabeculae of the mature pre-spawned female gonad. It was only located within certain cells, which may function to store aELH with the maturing gonad. It is postulated that a stimulus may act to release the hormone that acts directly on the smooth muscle of the gonad to release ova. Evidence from *Drosophila* shows that when a male mates and releases sperm, he also deposits an egg-laying hormone that induces egg-laying in the female (Park & Wolfner 1995). This hormone is related to the ones found in gastropods and other invertebrates.

The large amount of aELH expressed within the reproductively pre-spawned mature female may be a function of the gastropod requirement to release high concentrations of hormone into the hemolymph. It is known in molluscs that hormone release areas are high in number (Joosse 1979).

The antibodies we have produced can now be extended to further studies. They could be used to determine site-specific expression of aELH during the reproductive cycle *of H. rubra*, and other abalone species. Preliminary tests have indicated that they do react with the aELH in *H. asinina*. It would be interesting to determine if bioassays of gonads could quantify the presence aELH sufficiently to indicate spawning readiness. We are already doing bioassays with the aELH to induce spawning.

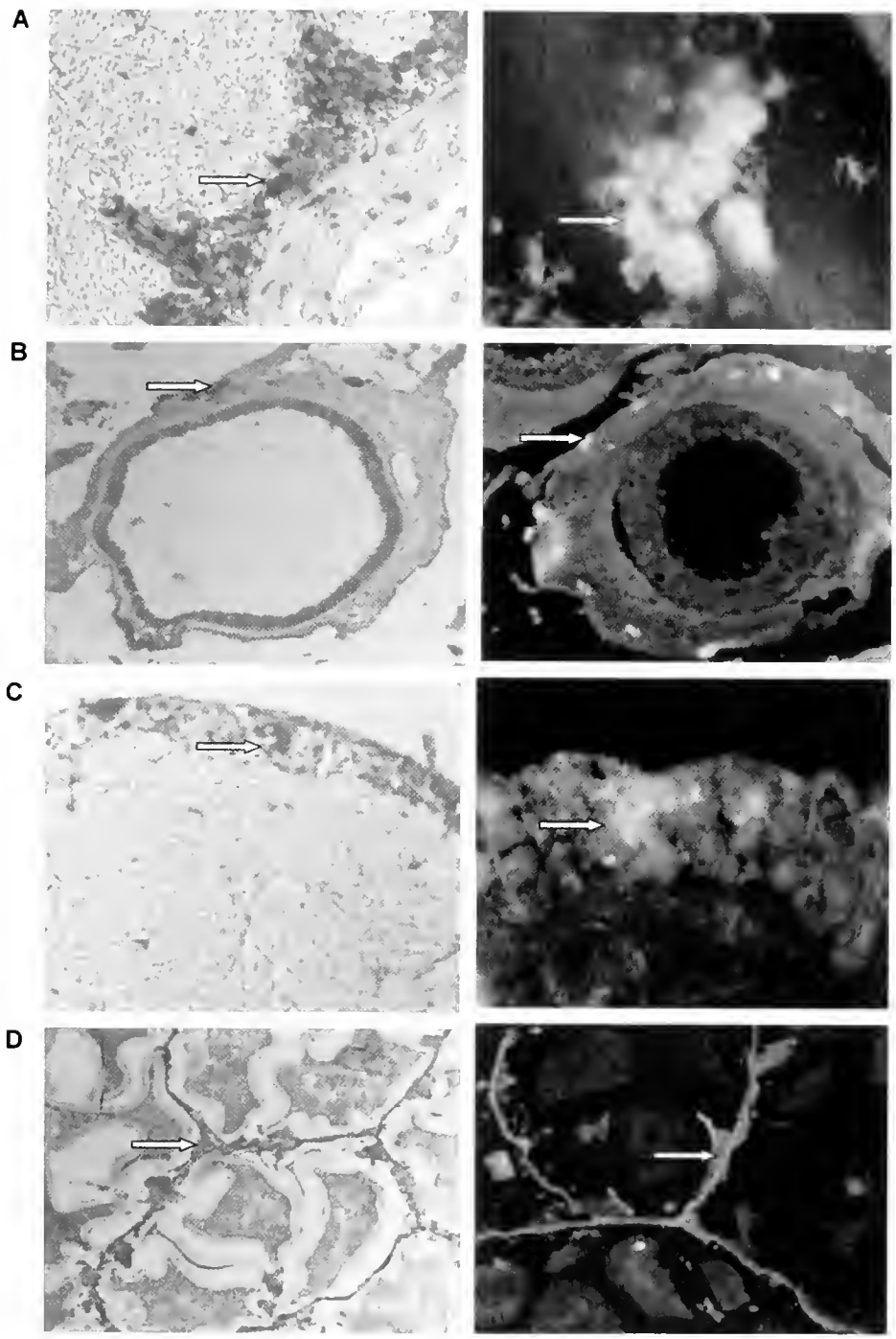

Figure 3. Localization of aELH in tissues of *H. rubra* using immunocytochemistry staining. Left: Tissues incubated with murine anti-aELH prior to detection using a DAKO LSAB2 kit to produce brick-red precipitate. Right: Tissues incubated with murine anti-aELH prior to detection using secondary incubation with FITC-conjugated goat anti-murine Ig to produce a green immunofluorescence under UV. A, immunoreactivity of aELH in cerebral ganglia; B, immunoreactivity of aELH in cells of a pleuro-pedal ganglia; C, immunopositive material around statocysts; D, immunopositive cells in the trabeculae of mature female gonads.

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MORPHOFUNCTIONAL STUDY OF THE HEMOCYTES OF *HALIOTIS ASININA*

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ABSTRACT The hemocytes of abalone (*Haliotis asinina*) were studied by light and electron microscopy in order to describe their main morphological features and to relate these to their role in immune defense. The cells are comprised of two differentiated types: agranulocyte or hyalinocyte and granulocyte. The hyalinocyte is characterized by the presence of several filopodia, a large nucleus with dense chromatin, a moderate amount of cytoplasm, microfilaments, oval and round-shaped mitochondria with rather dense matrix, a considerable amount of rough endoplasmic reticulum, few cytoplasmic granules, coated pits and vesicles, phagocytic vacuoles, and numerous large and small vacuoles. Like the hyalinocyte, the granulocyte possesses similar cytoplasmic organelles but in fewer number, and a peripheral organelle-free zone containing numerous dense granules of various types. The shape of the granules varies from round to oval to elongated forms. Several dense granules exhibit a crystalloid substructure that show close relationship to the plasma membrane. The average size of granulocytes is 9.68 ± 1.12 μm and hyalinocytes is 8.65 ± 0.77 μm.

KEY WORDS: *Haliotis asinina*, hemocytes

INTRODUCTION

Approximately 75 species of abalone have been reported in the world. Thailand harbors three species, namely, *Haliotis asinina*, *H. ovina*, and *H. varia* (Nateewatana and Hylleberge 1986, Tookvinart et al. 1986, Nateewatana and Bussarawit 1988). Among the three species, *H. asinina* is the biggest and has the most economic potential because of its large proportion of flesh and its good taste (Singhakriwan & Doi 1993). Due to the economic potential of this abalone species, a thorough understanding of the biology of *H. asinina* including its immune response is needed. In molluscs, hemocytes are involved in a variety of physiological and pathological functions including nutrient transport and digestion, wound and shell repair, internal defense, and exogenous and endogenous material excretion (Cheng 1981, Bayne 1983, Fisher 1986). In the literature, most of the morphological studies of molluscan hemocytes reported are of bivalve molluscs; only a few exist on abalone. There has been no study on the hemocytes of *H. asinina*. In this report, we studied the morphology of *H. asinina* hemocytes by using light and electron microscopy.

MATERIALS AND METHODS

Hemolymph was withdrawn from the cephalic arterial sinus of individual abalone and pooled. Pooled hemolymph was immediately poured into cold 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4, at 4°C, overnight. The hemocytes were centrifuged at 800 × g for 10 min at 25°C. The hemocyte pellets were post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate

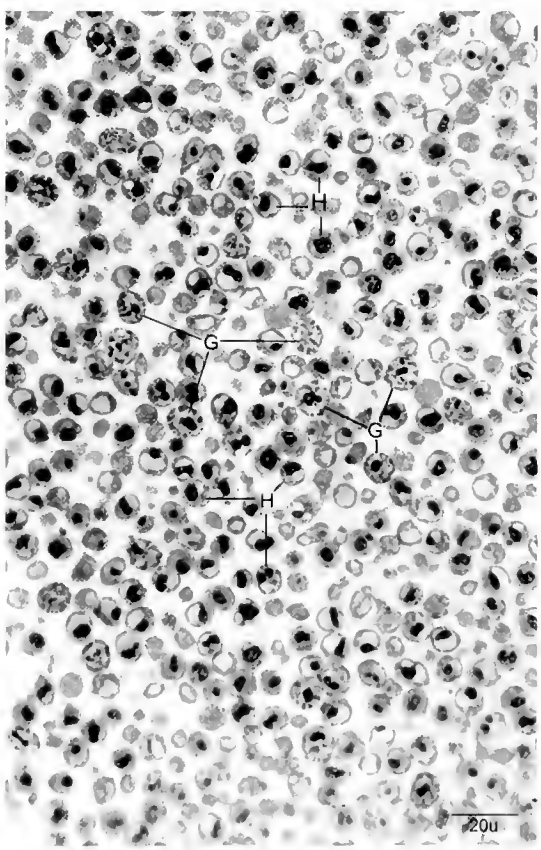

Figure 1. Semi-thin section of hemocytes of *H. asinina*, methylene blue stain. At this magnification, granulocytes (G) and hyalinocytes (H) are clearly differentiated.

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buffer, at 4 C, for an additional 2 h. They were then washed with sodium cacodylate buffer, dehydrated in a graded series of alcohol, cleared in propylene oxide and embedded in Aradite 502 resin. Blocks were sectioned at one-micron thickness by an ultramicrotome and stained with methylene blue for light microscopic observation. Ultrathin sections were cut and stained with uranyl acetate and lead citrate and viewed by Hitachi TEM H-300 at 75 kV.

RESULTS

Light Microscopy

Micrographs of the semithin sections of the *H. asinina* hemocytes are shown in Figures 1 and 2. Two populations of hemocytes, granulocytes and agranulocytes or hyalinocytes, were observed based on the presence and the absence of cytoplasmic granules. The proportions of granulocytes and hyalinocytes are 11.43% and 88.57% in this present study, respectively.

Granulocytes are characterized by the presence of numerous cytoplasmic granules. The granules tend to be arranged at the periphery of the cell, thus leaving the clear zone around the nucleus. Most of the cells are spherical or slightly oval, the largest measured 13.15 μm (9.68 ± 1.12 μm in diameter on average) with some filopodia extending from the plasma membrane. The nucleus is round to oval shaped and centric with a rather small nucleus/cytoplasmic ratio. The maximum nuclear size is 4.70 μm with an average of 3.70 ± 0.59 μm. Some nuclei are bilobed, elongated and indented.

The agranulocytes or hyalinocytes are spherical or oval. They are characterized by large nuclei and contain one or two prominent clear cytoplasmic zones. The cells are only slightly smaller than the granulocytes. The largest cell measured 11.22 μm in diameter and the average size is 8.65 ± 0.77 μm. Similar to the granulocytes, the nuclear profiles were round or oval, with some bilobed and indented. Nuclei were both eccentrically and centrically placed. The maximum nuclear size of the hyalinocyte is 5.98 μm with an average of 4.70 ± 0.60 μm. The nucleus/cytoplasmic ratio was relatively larger than that of the granulocyte. Filopodia were clearly visible extending from the cell.

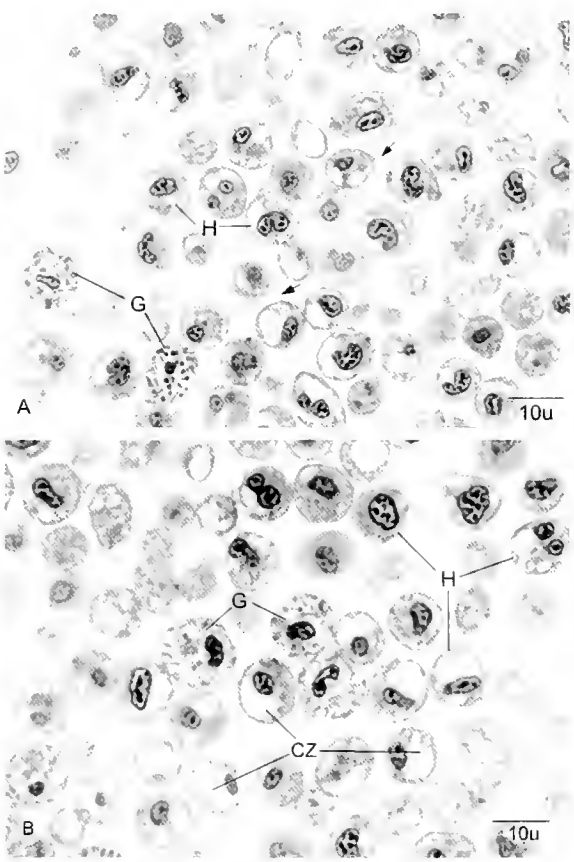

Figure 2. A–B. At higher magnification, more details are seen. Note the peripheral distribution of the granules and perinuclear clear area in the granulocytes, while large cytoplasmic clear zones (CZ) are seen in the hyalinocytes. Different shape, size and location of the nucleus in both granulocytes and hyalinocytes can be observed. Note also the filopodia (arrow) in both cell types.

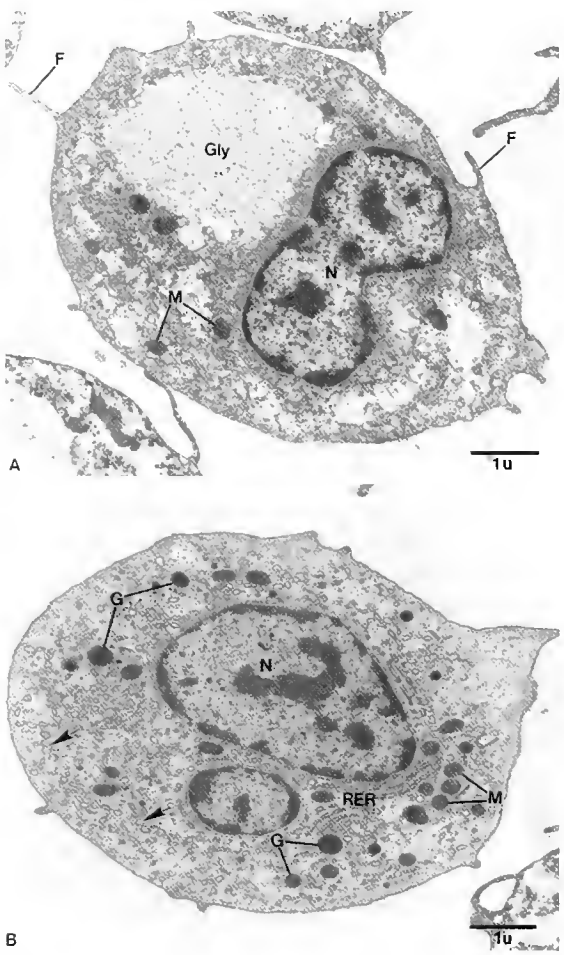

Figure 3. Electron micrographs of a hyalinocyte. A. Hyalinocyte showing bilobed nucleus (N) with moderate amount of heterochromatin. A lake of glycogen (Gly) is prominent. Few mitochondria (M) are seen. F, filopodia. B. In contrast to figure A, this hyalinocyte does not contain as prominent a glycogen area. There are few small round electron dense granules (G), rough endoplasmic reticulum (RER) and mitochondria (M). Note the presence of small vesicular bodies (arrow).

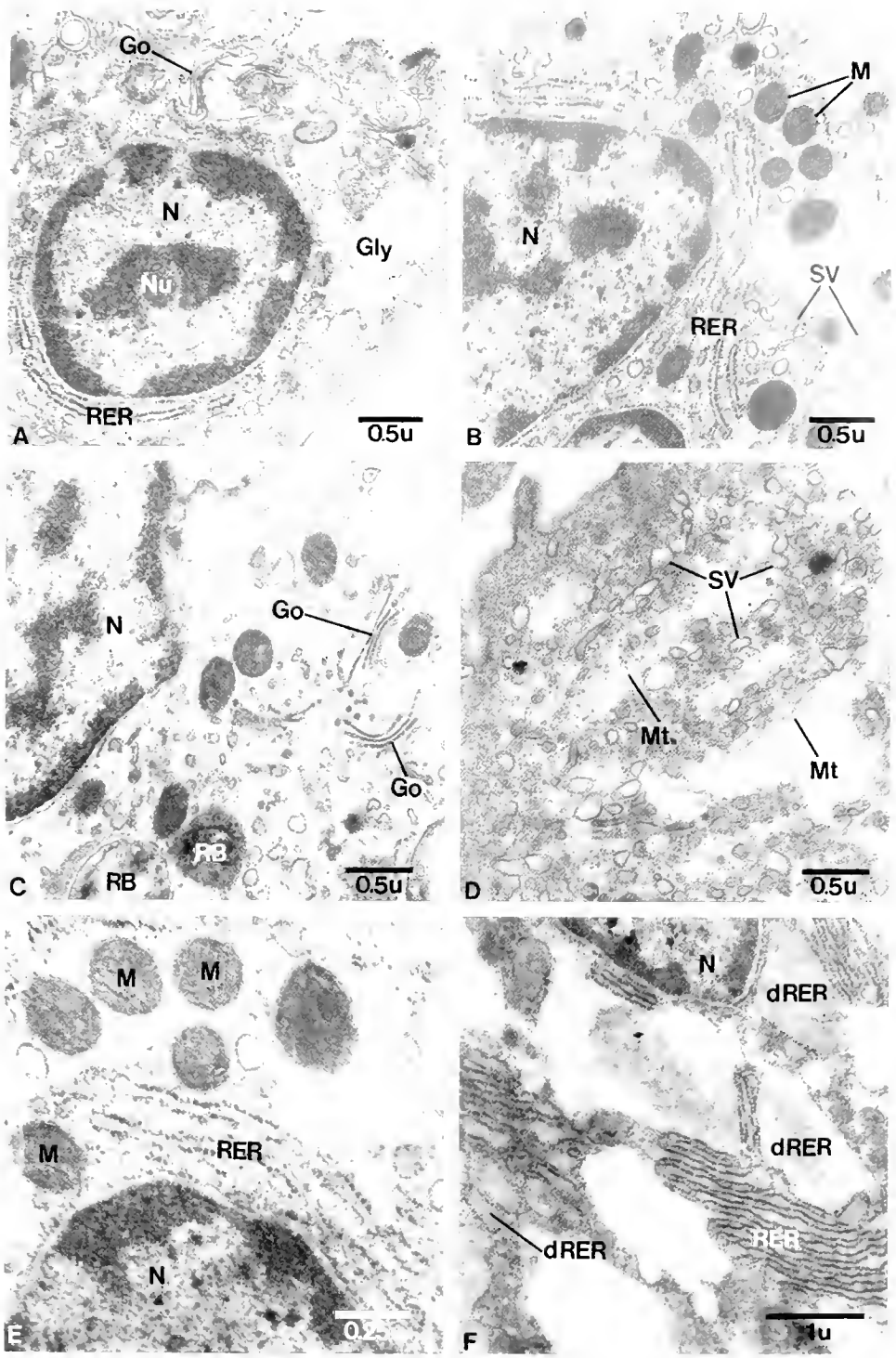

Figure 4. A series of electron micrographs showing details of some hyalinocyte organelles. A. Hyalinocyte with spherical nucleus (N) containing a moderate amount of chromatin and a prominent nucleolus (Nu). Note the presence of a glycogen area (Gly), Golgi apparatus (Go), and rough endoplasmic reticulum (RER). PM, plasma membrane. B. Hyalinocyte showing profiles of oval shape mitochondria (M) with rather dense matrix. Several small vesicles (SV) are also seen. M, mitochondria. N, nucleus. RER, rough endoplasmic reticulum. C. Well developed Golgi apparatus (Go) and residual bodies (RB) are seen here in this electron micrograph. Go, Golgi apparatus. N, nucleus. RB, residual body. D. Microtubules (Mt) and small vesicles (SV) are seen in the cytoplasm of the hyalinocyte. Mt, microtubule. SV, small vesicle. E. Mitochondria (M) with rather dense matrix are shown here with rough endoplasmic reticulum (RER). N, Nucleus. F. Hyalinocyte showing stack of rough endoplasmic reticulum (RER) and a few dilated endoplasmic reticulum (dRER) with light density amorphous material within cisternae. N, nucleus, RER, rough endoplasmic reticulum.

Electron Microscopy

The hyalinocyte is characterized by the presence of several filopodia, a large nucleus with dense chromatin and a moderate amount of cytoplasm Fig. 3. The mitochondria are round or oval in shape with rather dense matrix. A considerable amount of rough endoplasmic reticulum, Golgi apparatus, few cytoplasmic granules mostly of round shape, coated pits and vesicles, phagocytotic vacuoles, numerous large and small vacuoles including microfilaments were observed Fig. 4. When viewed by electron microscopy, the clear cytoplasmic zones observed by light microscopy corresponded to the areas composed mostly of unstained glycogen with some visible glycogen particles within. In some cells the pools of glycogen are quite large and seem to push the nuclei to the periphery.

Like hyalinocytes, the granulocyte possesses similar cytoplasmic organelles but in fewer numbers and a peripheral organelle free zone containing cytoplasmic granules of various shapes and sizes and various densities Fig. 5. The shapes of the granules vary from round, to oval to polyhedral elongated forms. The maximum size of the granule is 0.78 μm and the average size is 0.34 ± 0.11 μm. However, most of them are polyhedral elongated and only a few are spherical or oval. The close relationship of the granules and plasma membrane is noted. Some of the granules protruded from the plasma membrane Fig 6, while others coalesced or fused with the cell membrane. The nucleus is relatively small in size, round and located either eccentrically or centrically.

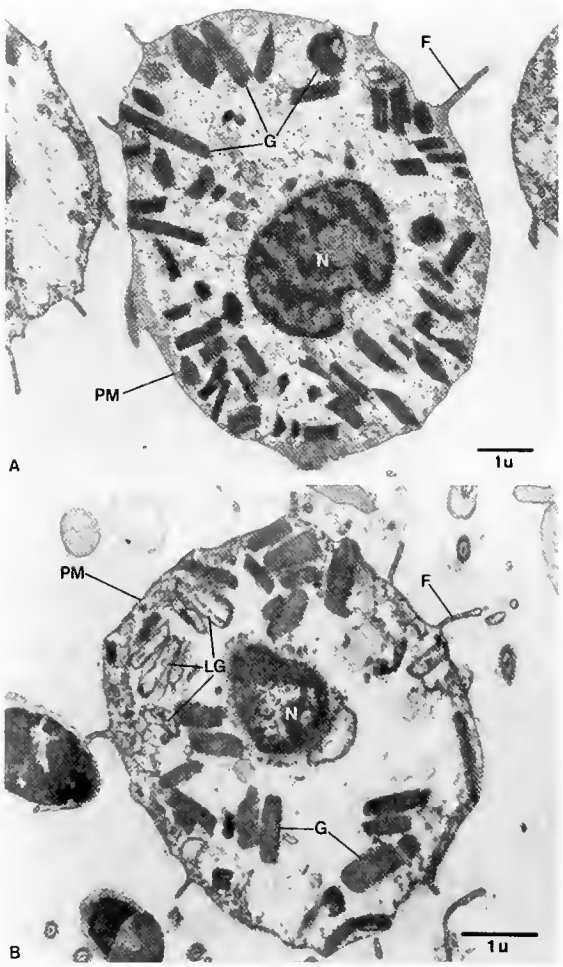

Figure 5. Electron micrographs of a granulocyte. A. Granulocyte showing different shapes and sizes of its granules. Note the peripheral distribution of the granules (G), several of them in close relationship with the plasma membrane (PM). As in hyalinocytes, the oval shaped nucleus contains moderate amount of heterochromatin. This cell is almost devoid of recognizable organelles. F, filopodia, N, nucleus. B. Variation of intragranular densities are illustrated in this micrograph from marked to light electron dense. The light granules (LG) contain intragranular inclusion bodies. F, filopodia, N, nucleus, PM, plasma membrane.

DISCUSSION

In this preliminary study on the hemocytes of the *H. asinina*, were composed of two differentiated cell types, the granular and the agranular or hyalinocytes. Cheng (1981) suggested that hemocytes should be designated as granulocytes and hyalinocytes. By using different techniques such as phase contrast microscopy, several fixatives and stains including electron microscopy, Foley and Cheng (1972), Foley and Cheng (1974), Cheng and Foley (1975), identified another cell type in the hemolymph of the bivalve mollusc, the fibrocyte. Fibrocytes were also described in the black abalone, *Haliotis cracherodii* (Shields et al. 1997).

In this study, we have shown that hyalinocytes contained very prominent areas or aggregates of glycogen. Cheng and Cali (1974) however reported that the granulocytes not the hyalinocytes contained a large aggregate of glycogen in the cytoplasm. This is in contrast to our findings in which we found glycogen primarily in the hyalinocytes Fig. 3B.

Of interest, is the observation of the close relationship of the granules of the granulocyte to the plasma membrane. Several granules protruded from the membrane and some fused with the membrane. This represents the process of releasing the content of the granules or most likely the lysosomes into the serum. The migration of the lysosome to the cell membrane and the extrusion from the granulocytes in *Mercenaria mercenaria* as evidenced by scanning and transmission electron microscopy were reported by Mohandas et al. (1985) and Mohandas and Cheng (1985). Fewer organelles observed in granulocytes indicate that when a cell becomes fully differentiated, reduction in the number of organelles accompanies this process.

This present study shows for the first time, the presence of two main types of hemocytes in *H. asinina*: hyalinocytes and granulocytes. We believe that the granules are the lysosomes based on the marked morphological resemblance to the lysosomal granules observed in the eosinophil. The particular function of each population in the defence mechanism in this species of abalone will be further studied.

ACKNOWLEDGMENT

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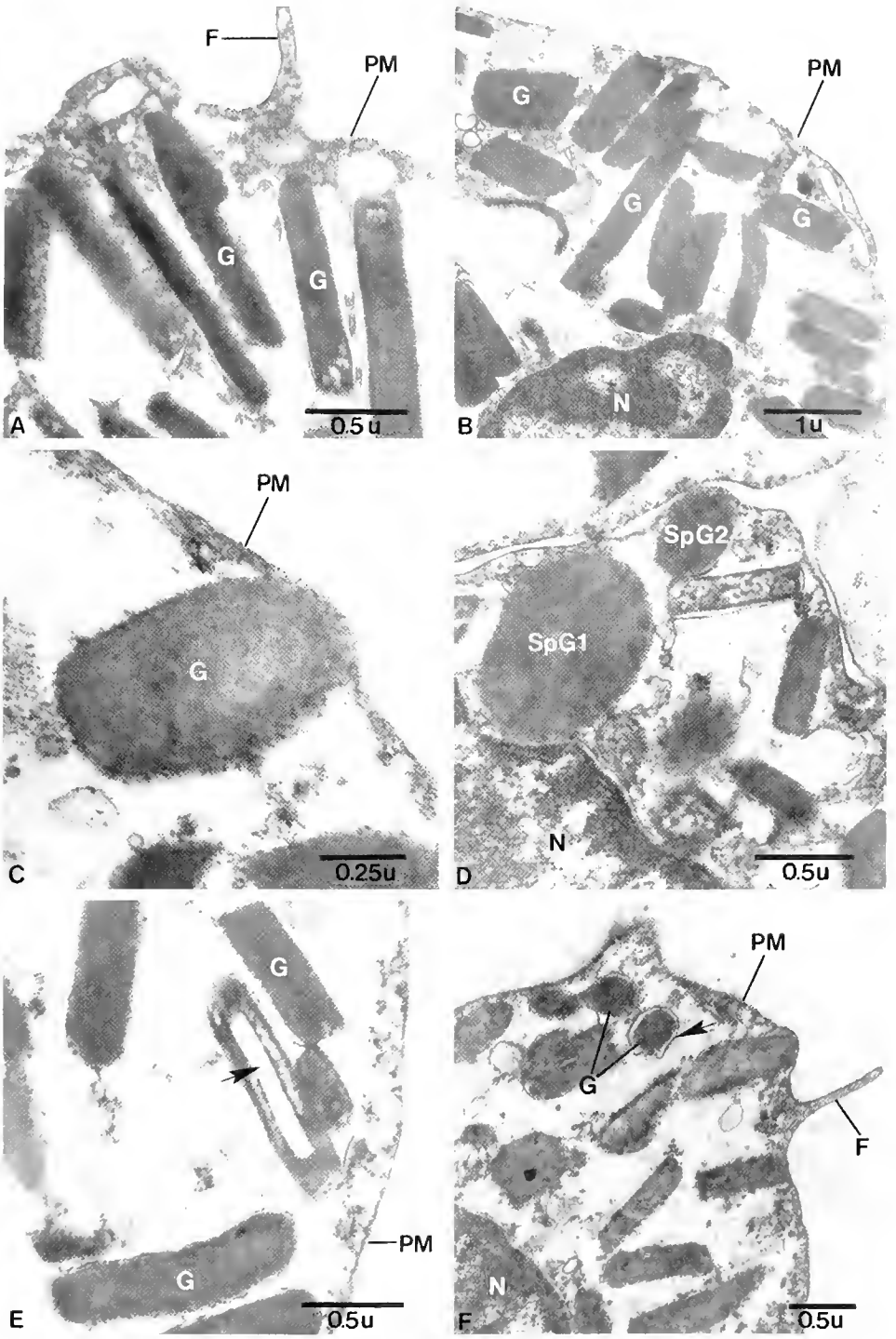

Figure 6. Variations in shape, size, density and the relationship to the plasma membrane of the cytoplasmic granules in the granulocyte of *H. asinina* are illustrated in details in this series of electron micrographs (A-F). A. Elongated polyhedral forms. F, filopodia. G, granule. PM, plasma membrane. B. Another view of the cytoplasmic granules of the granulocyte. G, granule. N, nucleus. PM, plasma membrane. C. Fusion of one of the granules (G) with the plasma membrane (PM). D. One large and one small spherical granule (SpG1 and SpG2) is seen. Note the heterogenicity of the elongated form granules, some of which contain intragranular inclusions. E. Picture frame like granule with hollow core is seen here (arrow). G, granule, PM, plasma membrane. F. Protrusion of one of the granules is evident here. Note the limiting membrane (arrow) around the small round shaped granule (G). N, nucleus. PM, plasma membrane.

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CHARACTERIZATION OF TRABECULAR CELLS IN THE GONAD OF *HALIOTIS ASININA* LINNAEUS

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ABSTRACT Trabeculae are the connective tissue sheets that extend perpendicularly from the outer capsules of both the testis and ovary to make contact at their innermost ends with the inner capsules separating the gonad from the hepatopancreas. Thus they divide the gonad into small compartments, and each trabecula forms the axis for individual spermatogenic or oogenic units, from which maturing germ cells are generated. When studied by light and electron microscopes, each trabecula is composed of central capillaries surrounded by muscle cells, collagen fibers, fibroblasts, and granulated cells that contain large rugby-shaped electron dense granules about 270 × 550 nm in size. The granulated cells branch extensively, and their processes become closely associated with bundles of nerve fibers that contain two types of granules, i.e., electron-dense and electron-lucent spherical-shaped granules with a diameter of about 165 nm and 150 nm, respectively. Thus, bundles of nerves and branches of granulated cells provide profuse innervation of the capsules and trabeculae. The granulated cells may be endocrine cells of the gonad which produce certain gonadotrophic factors yet to be identified.

KEY WORDS: *Haliotis asinina*, gonad, capsules, trabeculae, connective tissue cells, endocrine cells

INTRODUCTION

Studies on endocrinology of reproduction in molluscs present a number of interesting challenges because of a great variability of existing patterns. Most detailed studies of the modes of reproduction and corresponding endocrine controls have been carried out in *Aplysia* spp. (subclass Opisthobranchiata) and *Lymnaea* spp. (subclass Pulmonata) (Joose 1979, Joose 1988). In Prosobranchiata, including *Haliotis* spp., little is known about the reproductive hormones and their cellular origins. In molluscs studied so far, neurosecretory cells that are the putative sources of reproductive hormones, such as egg-laying hormone, are mostly localized in cerebral, pleuropedal and visceral ganglia (Bern & Hagadorn 1965, Dorsett 1986, Hahn 1994). The gonad represents another site where hormones controlling reproduction may be produced, as seen in the cases of Leydig cells and Copora luteal cells that lie in the connective tissue scaffold of vertebrates' gonad.

Our previous observations indicated a close association between cells in the connective tissue scaffold of the gonad of *H. asinina* and gamete cells during their development in various phases of the reproductive cycle (Sobhon et al. 1999). There are several types of cells present in the connective tissue of the gonad, including highly granulated cells with structural characteristics resembling those of endocrine cells (Apisawetakan et al. 1997, Sobhon et al. 1999). These observations together with the report by Chanpoo et al. (2000) strongly imply that reproductive hormones, such as egg-laying hormone, may also be produced intramurally within the connective tissue of the gonad by certain granulated cells. Moreover, there could be other cell types that are involved in the development of germ cells in the gonad that could evolve from the connective tissue compartment. Therefore, in the present study we have characterized various types of cells in the connective tissue scaffold of the gonad and attempted to define their possible functions.

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MATERIALS AND METHODS

Collection of H. Asinina Specimens

Adult *H. asinina*, reared in the land-based culture system, were provided by the Coastal Aquaculture Development Center, Prachaubkirikhun province, Thailand. They were cultured in concrete tanks that were well flushed with a mechanically circulated water and air delivery system to maintain a stable environment. Seawater was pumped directly from the nearby bay and passed through subsand filter before use (Singhagraiwan & Doi 1993). The animals were fed a diet of macroalgae (usually *Gracilaria* spp. and *Laminaria* spp.), supplemented with artificial food.

Specimen Preparation

Gonads were cut into small pieces, fixed in Bouin's fluid, and prepared for light microscopic examination by the paraffin method. For sem-ithin sections and TEM studies, specimens were fixed in a solution of 3% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.8 at 4°C overnight, postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer at 4°C for 2 h, then dehydrated in graded series of ethanol, cleared in propylene oxide, infiltrated and embedded in Araldite 502 resin, which was finally polymerized at 30°C, 45°C and 60°C for 24, 48 and 48 h, respectively. The specimens were then semi-thin-sectioned at one-micron thickness in a Porter Blum MT-2 ultramicrotome, stained with Methylene Blue or PAS, and observed in an Olympus Vanox light microscope. Ultrathin sections were cut and stained with lead citrate-uranyl acetate and viewed under a Hitachi TEM H-300 at 75 kV.

RESULTS

Connective Tissue Scaffold of the Gonad

The connective tissue frameworks that support the gonad in *H. asinina* consist of the outer capsules surrounding the ovary and testis, and the inner capsules that separate the hepatopancreas from the surrounding gonadal tissues. Flat connective tissue sheets, called

trabeculae, extend perpendicularly from the outer gonadal capsules through the interior of the gonads to touch the inner capsules. As a result, the gonads are partitioned into small incomplete compartments (Fig. 1A and B). Each trabecula acts as the axis on which early and growing germ cells are closely attached (Fig. 1C and D), thus giving rise to a discrete gametogenic unit representing, perhaps, a clone of germ cells which may arise from a single group of gonial cells. In each trabecula, muscle cells (Fig. 2A and D) lying alongside the central capillaries and fibroblasts together with collagen fibrils form the trabecular core. Granulated cells, which may be endocrine cells, are distributed in rows along both sides of each trabecula, separated from the gonadal compartment by thick basal laminae (Fig. 4A–C).

The Gonadal Capsules

The connective tissue of the trabeculae blends imperceptibly with those of the outer and inner capsules. The outer gonadal capsule is 40–50 μm thick and consists of 10 layers of cells (Fig. 2A and B). It is covered externally by a single layer of cuboidal or columnar epithelium, which consists of 2 types of cells. Type 1 are the principal cells which are tightly adhered to each other (Fig. 3A

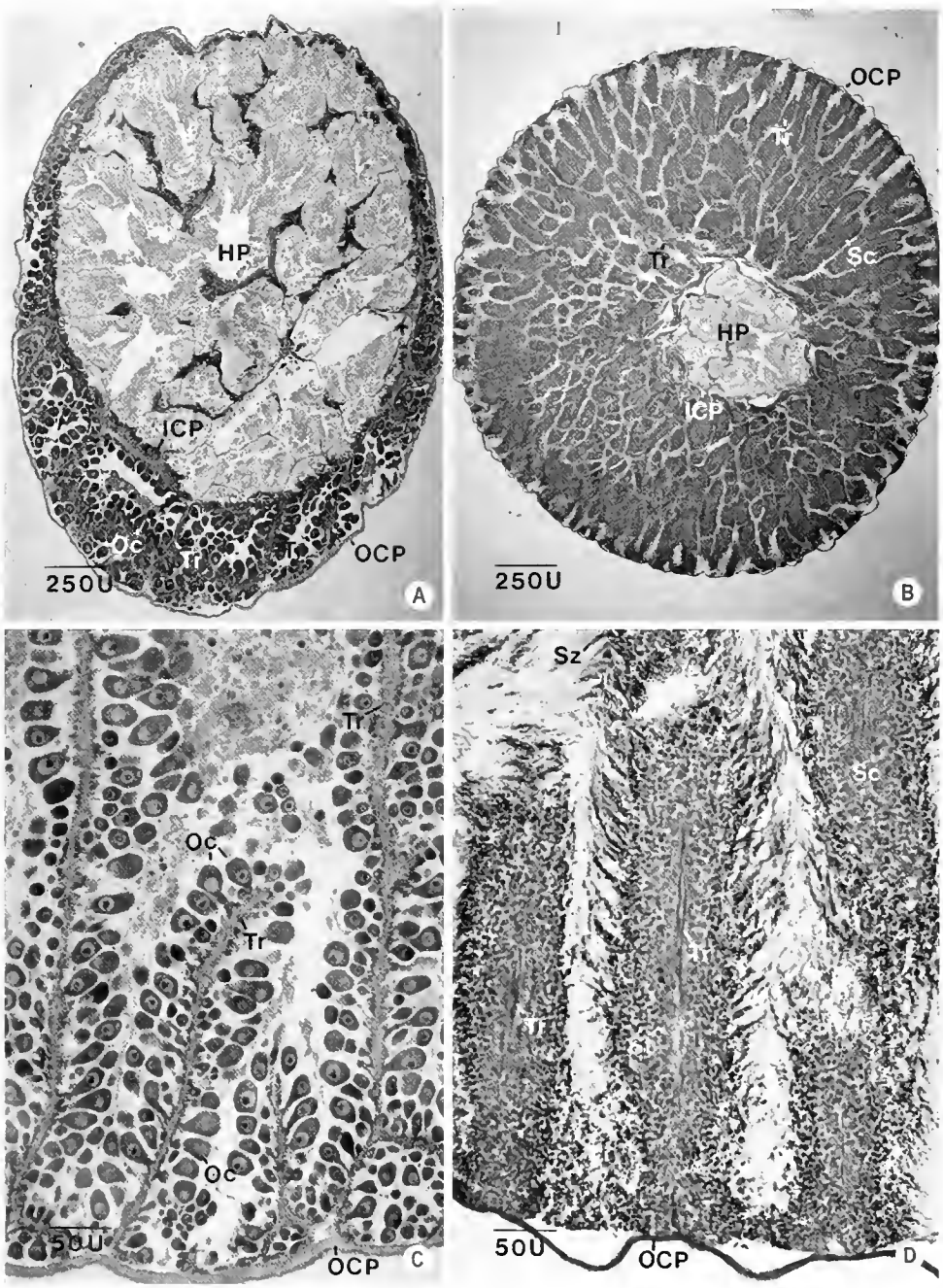

Figure 1. (A, B) Paraffin sections of an ovary (A) and testis (B) around the hepatopancreas (HP). Both organs are surrounded by outer and inner capsules (OCP, ICP), and trabeculae (Tr) form partitions between gonadal compartments. (C, D) Paraffin sections, showing oogenetic units of growing oocytes (Oc), and spermatogenic units of spermatocytes (Se), spermatids (St) and spermatozoa (Sz) around each trabecula (Tr).

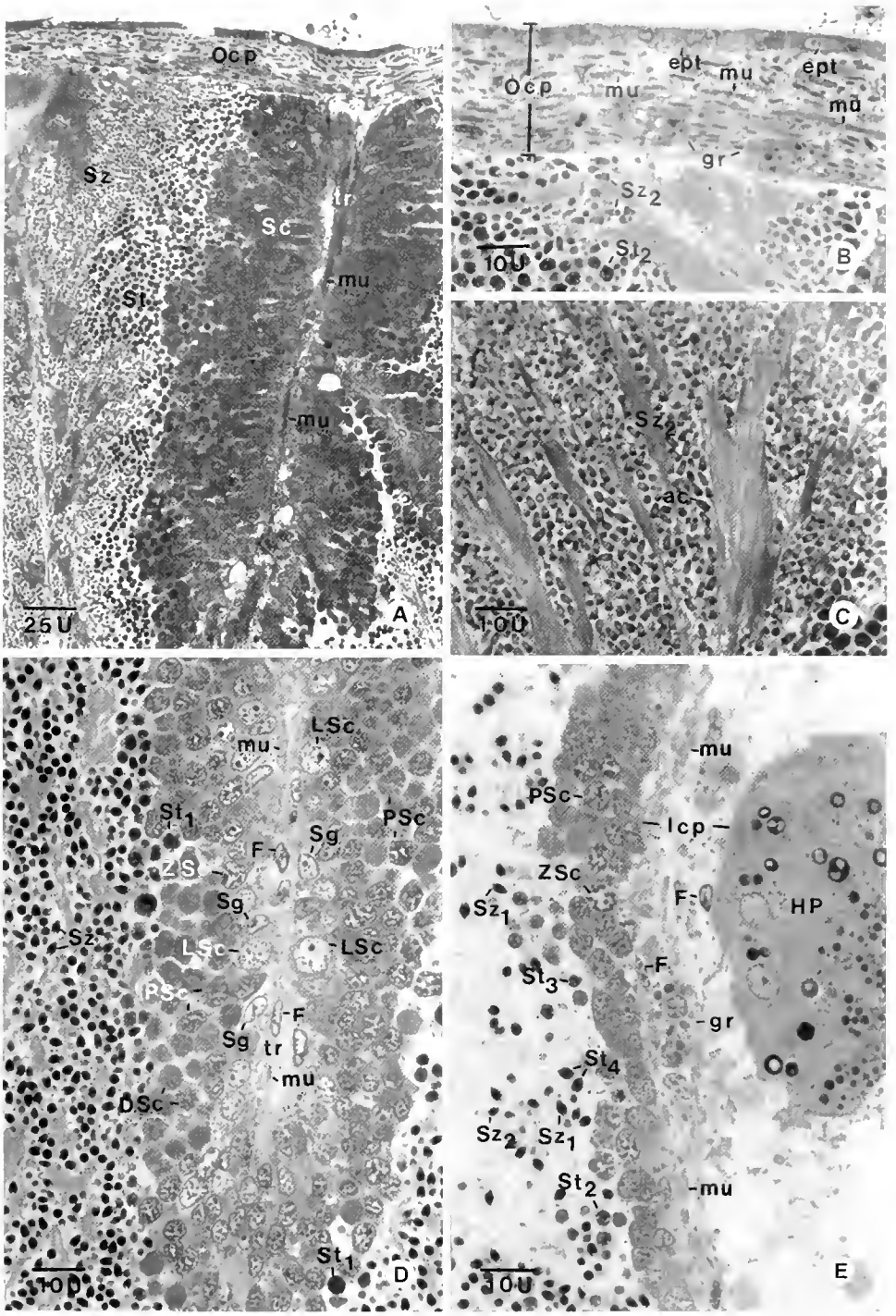

Figure 2. (A–C) Semithin sections of the testis stained with PAS, showing the outer capsule (OCP), and a spermatogenic unit around each trabecula (tr) (in A, B) and the accumulation of mature spermatozoa in the gonadal lumen (in C). In B, note that the outer capsule consists of cuboidal epithelium (ept) with alternate layers of muscle cells (mu) and collagen bundles. Granulated cells and their processes (gr) are present in large numbers. (D, E) Semithin sections of the testis stained with methylene blue, showing cellular components of trabeculae (in D) and the inner capsules (in E-ICP), and various stages of spermatogonia, spermatocytes, spermatids and spermatozoa surrounding the trabecula (Sg, LSc, PSc, ZSc, SSc, St, Sz) (granulated cells = gr; fibroblast = F; muscle cells = mu).

and B). The nuclei of these cells are elongated and have a thin rim of heterochromatin along the inner facet of the nuclear envelopes, and prominent nucleoli. There are large connecting blocks of heterochromatin scattered throughout the inner part of the nuclei. The cytoplasm is filled up with rough endoplasmic reticulum, and the apical cytoplasm contains a few dense irregular bodies that appear like lysosomes. The outer surface of the cells bears numerous microvilli. Type 2 are the goblet-like cells, whose apical cytoplasm is filled with moderately dense spherical granules, each about 600–650 nm in diameter (Fig. 3A and C). The nuclei of these cells are

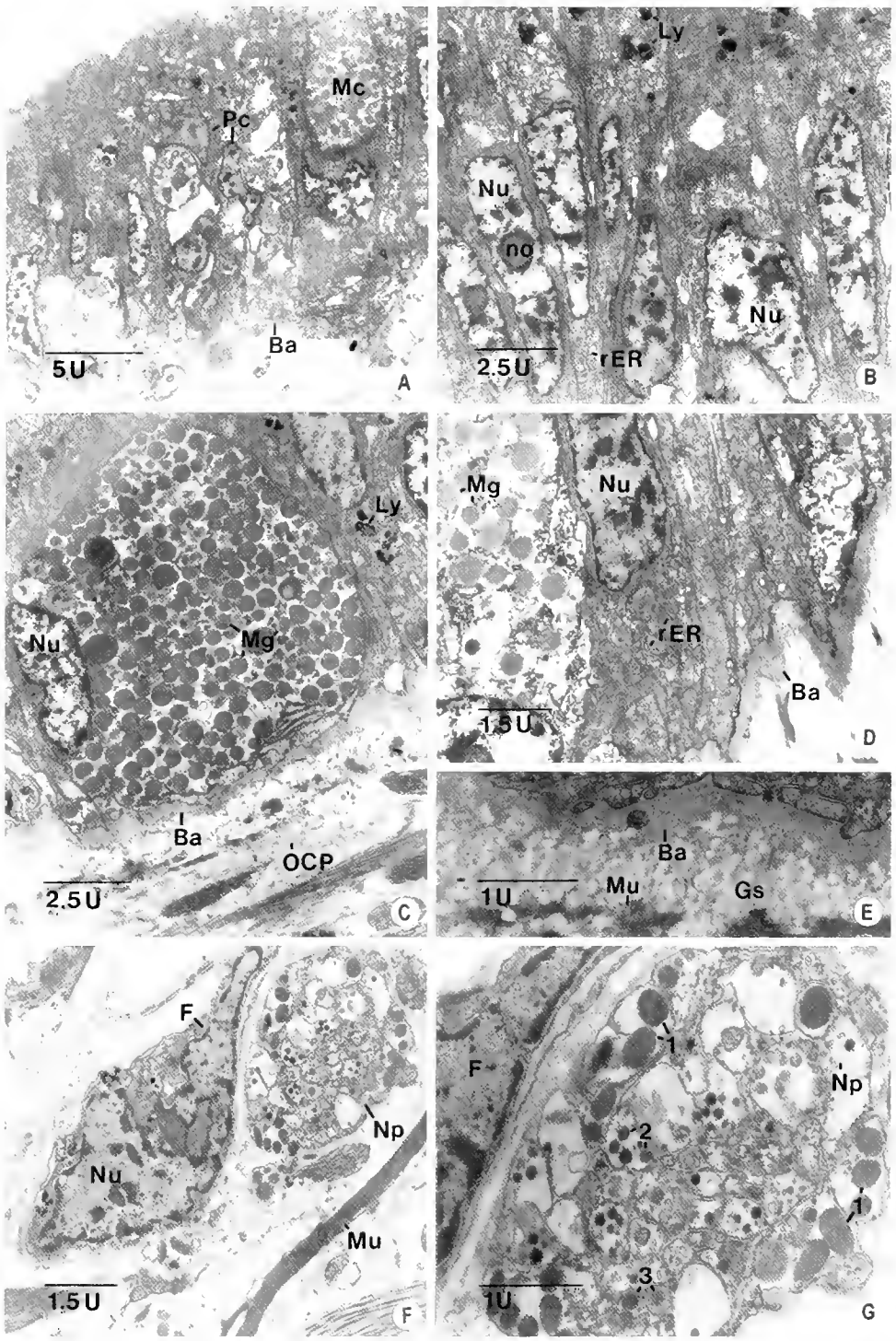

Figure 3. TEM micrographs of epithelium covering the surface of the outer capsule. (A–D) Principal cells (Pc) and mucous cells (Mc): the former are columnar or cuboidal-shaped cells tightly adhered together. Their cytoplasm contain numerous rough endoplasmic reticulum (rER) and lysosome-liked granules (Ly). Mucous cells contain abundant mucin granules (Mg) that fill up the apical cytoplasm. (E) The thick basal lamina (Ba) supporting surface epithelium, and layers of ground substance (Gs) and muscle cells (Mu). (F, G) Fibroblasts (F), and nerve bundles (Np) that innervate the outer capsule. In G, nerve processes containing dense and light spherical granules, about 165 and 150 nm in diameter (2, 3) respectively, are surrounded by processes of granulated containing large rugby-shaped granules about 270 × 550 nm in size (1).

eccentrically located toward the bottom, and they appear fairly similar to those of the first cell type. Both types of cells rest on a very thick basal lamina about 550 nm in width (Fig. 3A and D) which contains a meshwork of very fine filaments (Fig. 3E). Internal to the basal lamina are alternate layers of spindle-shaped muscle cells and the extracellular matrix which appears homogeneous and electron-lucent (Fig. 2B and Fig. 3E–3F). Among this extracellular matrix are fibroblasts, collagen fibrils, and small

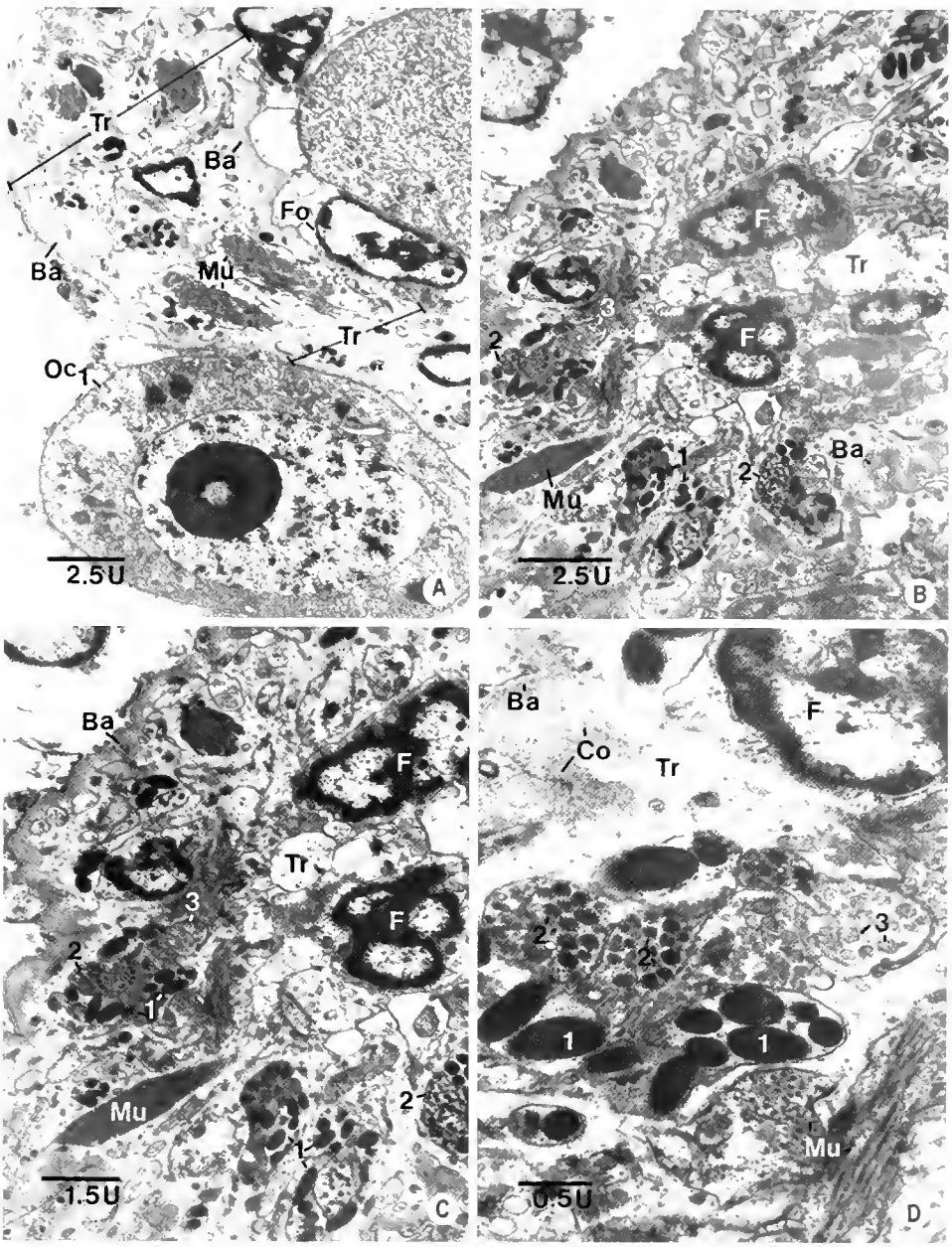

Figure 4. (A–D) TEM micrographs of a trabecula (Tr) in an ovary, which is separated from the gonadal compartment containing oocytes (Oc_1) by a thick undulating basal lamina (Ba). Within the core of each trabecula, there are collagen fibrils (Co), fibroblasts (F), muscle cells (Mu) and numerous processes of granulated cells (1) in close association with nerve processes (2, 3).

nerve bundles (Fig. 3F and G). The latter consist of nerve processes of various sizes, some of which contain electron-dense spherical granules (about 165 nm in diameter), while others contain more electron-lucent granules (about 150 nm in diameter). The peripheral part of each bundle is usually surrounded by branches of granulated cells that contain large rugby-shaped granules (about 270 × 550 nm in size). Most of the nerve bundles are situated close to the inner facet of the capsule, which is lined by a single squamous epithelium resting on another thick layer of basal lamina, that separates the capsule proper from the gonadal compartment (Fig. 2B). The inner capsule consists of a thin layer of loosely arranged connective tissues and muscle cells. It is separated from the gonadal compartment and hepatopancreatic tissue by basal laminae (Fig. 2E). The cellular constituents are composed of muscle cells, fibroblasts, and granulated cells which appear to be more abundant than in other areas of the connective tissue scaffold (Fig. 2E).

Trabecular Compartment

Each trabecula could be considered as a circumscribed compartment. As described earlier, this compartment is the continuation of the outer capsule, hence their main cellular compositions are muscle cells and fibroblasts embedded in the extracellular matrix and collagen fibrils. This compartment is partitioned off from adjacent gonadal compartments by thick convoluted basal laminae similar to those observed lining the outer and inner capsules (Fig. 4A–C). In addition, there are rows of granulated cells or their

branches at regular intervals along the trabecular sides of the basal laminae (Fig. 4A–C).

Muscle Cells

These cells are intermediate in characteristics between skeletal and smooth muscle cells of vertebrates. They have short thick filaments, each of which is surrounded by 12 to 15 long thin filaments. The thin filaments are attached to the dense bodies which are scattered throughout the cytoplasm, with some adhering to the plasma membrane (Fig. 5A–D).

Fibroblasts

These cells are similar in characteristics with their counterparts in vertebrates' connective tissues, and they are embedded within the collagen fibrils which they synthesize (Fig. 3F and Fig. 4B and C).

Granulated Cells

These cells have oval nuclei with thin rims of heterochromatin along the nuclear envelopes, and patches of heterochromatin in the center (Fig. 2E and 6A). They also have long processes that become closely associated with bundles of axons, which may come from neurons outside the gonads. As a result, there appear to be three types of granules within and around each bundle of axons and branches of granulated cells (Fig. 3F–G and Fig. 6C–F) similar to those found in the capsules. Isolated cytoplasmic branches of granulated cells containing groups of large granules are also frequently observed lying in rows near muscle cells (Fig. 5A and E), and under the undulating basal laminae that separate the trabeculae from the germ cell compartments (Fig. 4B–C and 6B). At many sites muscle cells appear in close apposition to solitary branches of granulated cells (Fig. 5E).

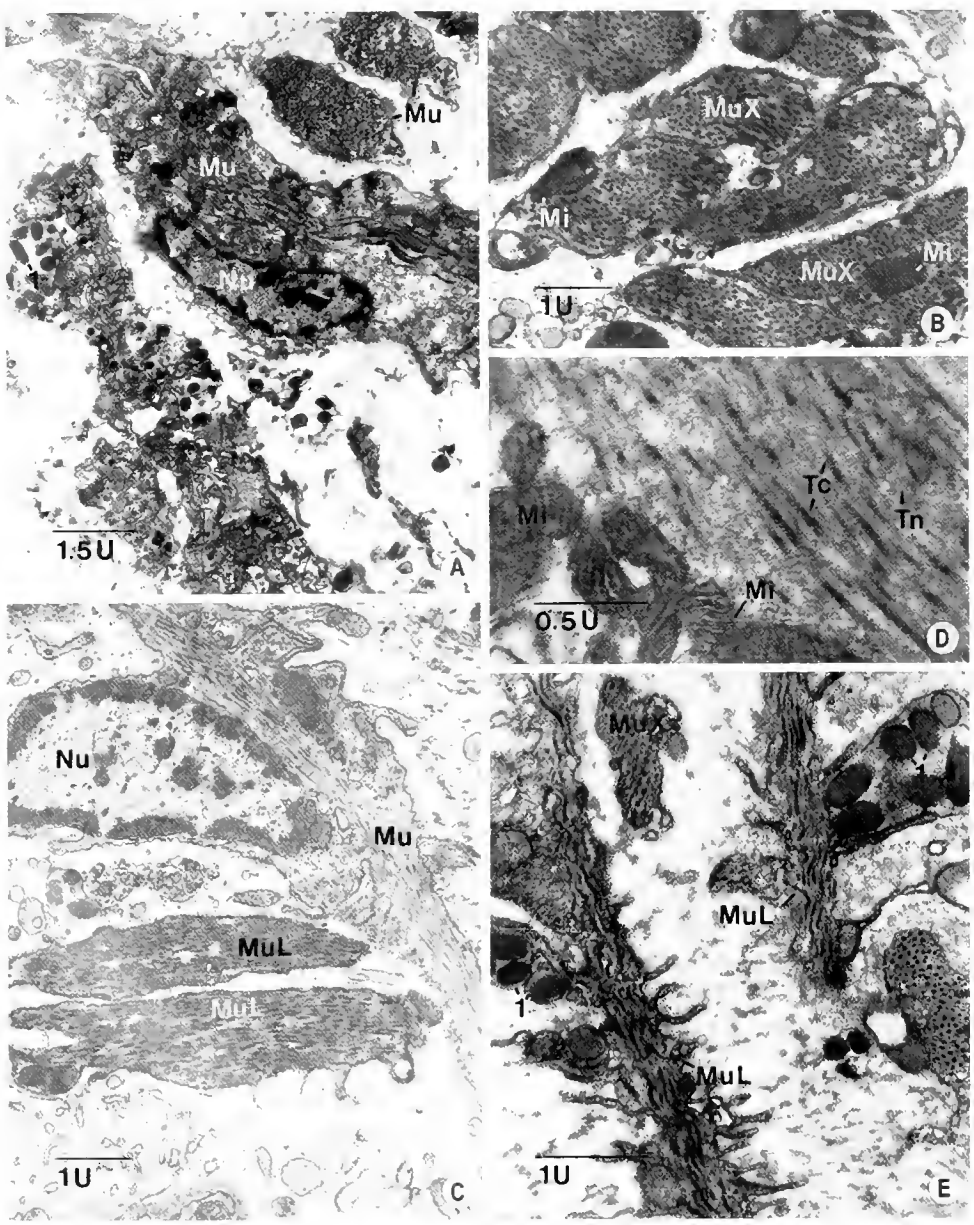

Figure 5. TEM micrographs of muscle cells, showing close association with the processes of granulated cells (1-in A, E). Cross (in B) and long sections (in C, D) of muscle cells, exhibit dense thick filaments (Tc) each of which is surrounded by numerous thin filaments (Tn). A large number of mitochondria (Mi) are also present in muscle cells.

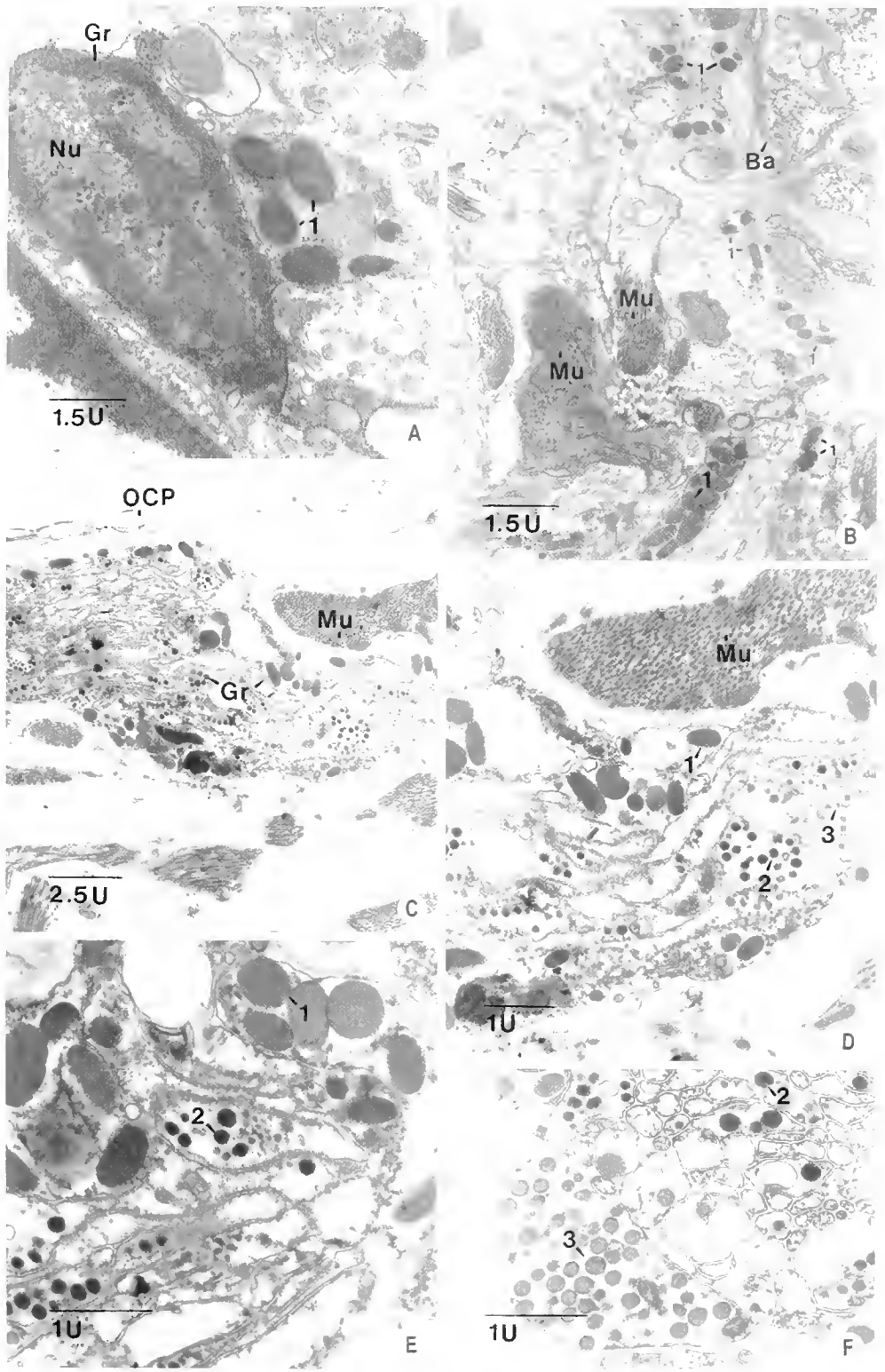

Figure 6. (A, B) TEM micrographs of a granulated cell (in A). The cell has an oval nucleus with patches of heterochromatin along the nuclear envelope and in the center. The cytoplasm contains large rugby-shaped granules about 270 × 550 nm in size, with an electron-dense matrix (1). In B, branches of granulated cells (1) are dispersed at intervals along the basal lamina (Ba). (C–F) The nerve bundles in trabeculae consist of two types of axons that contain dense (2) and light (3) spherical granules (about 165 and 150 nm in diameter). Processes of granulated cells (1) are closely associated with the periphery of each nerve bundle (in D, E).

DISCUSSION

The general histology of the gonad and classification of various types of germ cells in many species of abalone, such as, *H. tuberculata* (Stephenson 1924, Croft 1929), *H. discus hannai* (Tomita 1967, 1968), *H. cracherodii* (Webber & Giese 1969), *H. rufescens* (Young & DeMartini 1970, Martin et al. 1983), *H. diversicolor diversicolor* (Takashima et al. 1978), *H. asinina* (Apisawetakan et al. 1997, Sobhon et al. 1999) have been reported. However, most studies neglect the cellular compositions and detailed structure of the connective tissue scaffold of the gonad, apart from mentioning casually that the gonadal capsules and trabeculae are made of fibro-muscular tissues.

In our detailed studies using both light and electron microscopy, we found that the cellular compositions of connective tissue scaffold are more complex than previously thought. Within these connective tissue frameworks, which may be termed trabecular-capsular compartments, there are many types of cells that may be involved in the physiology of the gonad, including the production of reproductive hormone, and the release of mature gametes. These cells are muscle cells, fibroblasts and granulated cells.

The most striking feature is the presence of a large number of granulated cells, with large-endocrine like granules, in the trabeculae and capsules of the gonads. It is remarkable that these types of cells and their branches form an extensive network within the connective tissue scaffold of the gonad. Immunolocalization studies of abalone egg-laying hormone (aELH) performed by our group demonstrated that these cells could be one of the primary producers and/or storage sites of aELH (Chanpoo et al. 2000). Even more remarkable is that there are numerous nerve processes, which consist primarily of axons containing neurochemical vesicles, coming into close contact with branches of granulated cells. These bundles of nerve and granulated cell processes are mostly observed within the gonadal capsules. Judging from this appearance, gonads of *H. asinina* are highly innervated organs. In contrast to vertebrates, it is possible that the nervous system still play a more direct role in controlling the physiology of the gonad.

The muscle cells show typical features as present in most invertebrate species, and these characteristics are intermediate between skeletal and smooth muscle cells of vertebrates (Bennett & Threadgold 1973, Stitt et al. 1992). The thick filaments are short bundles consisting primarily of paramyosin protein (Ishii & Sano 1980) and each is surrounded by up to 12 to 15 thin filaments. This indicates that the muscle cells may be able to generate contractile force greater than that of vertebrate smooth muscle. It was found that branches of granulated cells, the putative egg-laying hormone producer, are lying close and frequently tightly adhered to the muscle cells. Immunolocalization study by our group demonstrated that aELH also binds to muscle cells in the capsules and cores of trabeculae (Chanpoo et al. 2000). It is, therefore, possible that aELH, upon being released from the granulated cells, may stimulate the contraction of muscles in the trabeculae and capsules of the gonad, and results in the release of mature gamete cells from the abalone at the time of spawning.

Fibroblasts and collagen fibrils appear to be similar in most respects to their counterparts in vertebrates' connective tissues. Thus, their functions could be primarily supportive.

ACKNOWLEDGMENT

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LOCALIZATION OF EGG-LAYING HORMONE IN THE GONADS OF A TROPICAL ABALONE, *HALIOTIS ASININA* LINNAEUS

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ABSTRACT Connective tissue frameworks of the gonad of *H.asinina* consist of the outer gonadal capsule, flat sheets of connective tissue, called trabeculae, that extend from the former toward the inner capsules separating the gonad from the hepatopancreas. Trabeculae, thus, partition the gonad into compartments; and each trabecula acts as the axis on which growing germ cells are attached and proliferate. Each trabecula contains small capillaries in the center, surrounded by muscle cells, collagen fibers intermingled with fibroblasts, and a substantial number of granulated cells that branch extensively. Localization of abalone egg-laying hormone (aELH) was performed by immunoperoxidase technique using polyvalent antibody against recombinant aELH as a probe. Anti-aELH exhibited strong bindings, which implied the presence of aELH, to muscle cells and granulated cells within trabeculae and capsules of both male and female gonads. The cytoplasm of immature oocytes (stages 1, 2, 3) were moderately stained, while that of mature oocytes (stages 4, 5) were only weakly stained. In contrast, male germ cells were not stained.

KEY WORDS: *Haliotis asinina*, gonad, capsules, trabeculae, connective tissue, egg-laying hormone

INTRODUCTION

Donkey's ear abalone, *Haliotis asinina*, is a commercially important abalone species in Thailand because of their maximum proportion of flesh, good taste and relative abundance in Thai coastal water (Singhagraiwan & Doi 1993). The high demand for this abalone has increased pressure on natural stocks, which needed to be maintained if the population is to remain sustainable. So far, most studies have been on practical aspects of finding the optimal aquaculture system while knowledge concerning the reproductive biology of this abalone has received little attention.

A number of gastropod species have been studied with respect to the effects of the egg-laying hormones on their reproductive activities. In *Aplysia californica*, an opisthobranch, egg laying was caused by the injection of the extract from bag cells of abdominal ganglion (Arch 1976, Kupfermann 1967). Administration of the extract from caudo-dorsal cells (CDC) of cerebral ganglia in *Lymnaea stagnalis*, a pulmonate, also caused egg laying (Geraerts & Bohlken 1976). In prosobranchs, injections of crude homogenates of pleuropedal and visceral ganglia could induce spawning in *H. discus hannai* (Yahata 1973). The peptide that activated egg laying in *Aplysia* spp. had been characterized and called egg-laying hormone (ELH) (Chiu et al. 1979), whereas it was called caudo-dorsal cell hormone (CDCH) in *L. stagnalis* (Ebberink et al. 1985), and abalone egg-laying hormone (aELH) in *H. rubra* (Wang & Hanna 1998). These egg-laying hormones may be related, judging from their amino acid numbers and compositions. ELH has 36 amino acids and a molecular weight of about 4.3 kD (Chiu et al. 1979), (in comparison to CDCH, which has molecular weight about 4.5 kD (Ebberink et al. 1985), and aELH about 4.3 kD) (Wang & Hanna 1998). Genes encoding ELH, CDCH and aELH have been cloned (Scheller et al. 1983, Vreugdenhil et al. 1988, Wang & Hanna 1998). The nucleotide sequence encoding aELH of *H. rubra* was found to have a 95.4% homology with that of CDCH in *L. stagnalis*, but only 51.9% homology with that of ELH in *A. californica*. Only one amino acid difference was found between aELH of *H. rubra* and CDCH of *L. stagnalis*, while there were 19 amino acid differences between aELH of *H. rubra* and ELH of *A. californica* (Wang & Hanna 1998).

It has been suggested that ELH acts directly on muscle of the capsules and trabeculae of the gonads to initiate egg laying and sperm release in the manner analogous to the action of oxytocin on myoepithelial cells of vertebrates (Coggeshall 1972). In abalone, it remains to be studied where this hormone is synthesized, and how it is distributed in the reproductive organs. In the present study, we have investigated the distribution of this hormone in the gonads of both male and female *H. asinina* by using mouse polyclonal antibody to recombinant aELH of *H. rubra* for immunohistochemical detections.

MATERIALS AND METHODS

Experimental Animals

Sexually-mature male and female *H. asinina* more than two years old were obtained from The Coastal Aquaculture Development Center, Prachuabkirikhun Province, Thailand, during the months June to July and August to September, which are the periods when the gonads enter the proliferate and maturing-spawning phases. These abalone were reared in a land-based aquaculture system by being kept in concrete tanks, which were well flushed with sand-filtered sea water, and aerated with a mechanical air delivery system. They were given appropriate algal food, usually *Gracilaria* and *Laminaria* spp., *ad libitum*, supplemented with artificial food, and kept under a normal daylight cycle.

Samples were taken from the gonads of at least five animals of either sex at both periods of collection. The tissues were prepared

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for histological examinations by paraffin-embedded and epoxy plastic-embedded (semi-thin) methods as used for transmission electron microscopy and for detecting the distribution of aELH by immunoperoxidase method.

Paraffin and Semi-Thin Methods

For histological studies, gonadal tissues were cut into small pieces, fixed in Bouin's fixative, dehydrated and embedded in paraffin, then sectioned at 5 μm thick and stained with hematoxylin-eosin or PAS-hematoxylin. For semi-thin sections, gonadal tissues were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, dehydrated in ethanol, cleared in propylene oxide, and then embedded in Araldite 502 plastics. Semi-thin sections at 1–2 μm were cut with the ultramicrotome and stained with methylene blue or PAS.

Immunoperoxidase Method

Frozen sections of gonadal tissues were fixed with acetone at −10°C for 10 min. After blocking endogenous peroxidase by immersing in 3% H_2O_2 for 15 min the sections were washed with 0.05 M Tris buffer saline (TBS), pH 7.6. The sections were overlaid in moist chamber with 0.1% glycine in 0.05 M TBS, and 4% BSA in 0.05 M TBS, for 30 min each, to block non-specific bindings. Then the sections were covered for 1 h with primary antibody consisting of mouse polyclonal antibody against recombinant *H. rubra* egg-laying hormone (Wang & Hanna 1998), with dilution 1:10,000. The control sections were incubated with 0.05 M TBS in place of primary antibody. After that the sections were incubated with secondary antibody, which is biotinylated rabbit anti-mouse IgG (Zymed Co., California, USA), with dilution 1:100, for 30 min. The sections were then washed in TBS, covered with the combination of Z-avidin and biotinylated peroxidase (Zymed Co.) in the same buffer, with dilution 1:100, for 30 min. Finally, the sections were immersed in the substrate solution containing 5 ml of 0.03% (w/v) 3,3′ diaminobenzidine (DAB) plus 17 μl H_2O_2, for 15 min. Finally, the sections were washed several times with distilled water, and some were counter-stained with hematoxylin before being mounted in buffered glycerol, and observed in an Olympus Vanox microscope.

RESULTS

Connective Tissue Frameworks

Connective tissue scaffolds of the gonads in either sex consist of the outer capsule which is made of several alternate layers of muscle and collagen bundles, lined on the outside by a single layer of cuboidal epithelium (Fig. 1A, B, F). On the inside the connective tissue of the capsule extends inwards to form sheets, called trabeculae, that have straight hemolymph capillaries running in their cores. Surrounding the capillaries are muscle cells, collagen fibrils, fibroblasts, and granulated cells (Fig. 1A, B, E, F). The interior ends of trabeculae are connected with the inner capsule made of a thin loose connective tissue layer that separates gonad from hepatopancreas. Thus, trabeculae divide the gonads into small compartments, and each trabecula forms the axis on which germ cells are attached and proliferate. Early stage cells, such as spermatogonia and oogonia are seen closely bound to the connective tissue of trabeculae. Middle stage germ cells, such as spermatocytes and oocytes, are more detached and appear further away from trabeculae. Mature cells including spermatozoa and stage 5 oocytes, which are the majority of cells found in the gonads in mature phase, are completely detached from the trabeculae and fill up the lumen of the compartments (Fig. 1A, B). Of special interest is the presence of granulated cells, whose soma are large and have an ovoid shape about 10 × 18 μm in size. Each cell has a small spherical nucleus that contains mostly euchromatin. The cytoplasm contains numerous dense spherical granules about 0.3–0.6 μm in size (Fig. 1C, D, E). In PAS stained sections, the granules exhibit PAS positive substance (Fig. 1C, D). The granulated cells may give off extensive branches since in many locations only cytoplasmic processes containing dense granules were observed (Fig. 1E, F). Generally, granulated cells are present in all areas of the connective tissue scaffolds; however, they tend to be more numerous in the outer and inner capsules of the gonads.

Immunoperoxidase Staining

By using the immunoperoxidase method, the presence of brownish staining suggested that there are strong and specific bindings of anti-aELH in the trabeculae and the capsules of the gonads in both sexes. The brownish stain is distinct on the overall content of trabeculae and capsules which delimit gonadal compartments of both the ovary and testis of the mature phase abalone (Fig. 3A–D, Fig. 4A, B), in contrast to the control sections which are completely unstained (Fig. 2A). Within each trabecula, there are more intensely stained spots or streaks, which at high magnification, appear to be muscle cells and granulated cells that contain large brownish granules in the cytoplasm (Fig. 3B, D, Fig. 4B, C, D). The latter cells are similar in appearance and general distribution with the granulated cells observed in paraffin and semithin sections, which exhibit better cellular morphology. The positively stained granulated cells were observed in both the outer and inner capsules and the trabeculae. However, there appear to be a higher concentration of these positive cells in the inner capsule that separates gonads from the hepatopancreas (Fig. 2B, C, Fig. 4A, B), in both proliferate (Fig. 2B–D) and mature phases (Fig. 4A–D) of the gonadal cycle. Connective tissue in the trabeculae and in the capsules are moderately stained, but they appear more intense in the mature than the proliferate phase of the gonadal cycle (Fig. 2B, 3A). In the ovary, the cytoplasm of early oocytes (stages 1, 2, 3), which are the majority of cells in the proliferate phase, is moderately stained (Fig. 2A, B), while the cytoplasm of mature oocytes (stages 4, 5) which the majority of cells in the mature phase, is only weakly stained (Fig. 3A, B, C). In contrast, there is no staining of either early male germ cells or spermatozoa in the testis (Fig. 4A, B, C).

DISCUSSION

The expression of the egg-laying peptide gene in the bag cells of the abdominal ganglion, atrial gland, and pleural ganglion of *A. californica* has been detected by *in situ* hybridization, using radiolabeled cDNA probes to ELH mRNA (McAllister et al. 1983). Some work had also been done to locate the ELH receptor within the ovotestis of *A. californica*. ELH binding-protein from the ovotestis was purified by ELH affinity column chromatography, and antibodies were produced for localizing this protein in *A. californica* gonads (Choate et al. 1993). It was revealed that the cytoplasm of oocytes is the only site of immunoreactivity, which was never detected in spermatocytes and spermatozoa. In *L. stagnalis*, *in situ* hybridization experiments with cDNA probes revealed a high level of CDCH mRNA expression in caudodorsal cells in the cerebral ganglia. The observed expression pattern correlated with immunocytochemical data which implied that CDCH

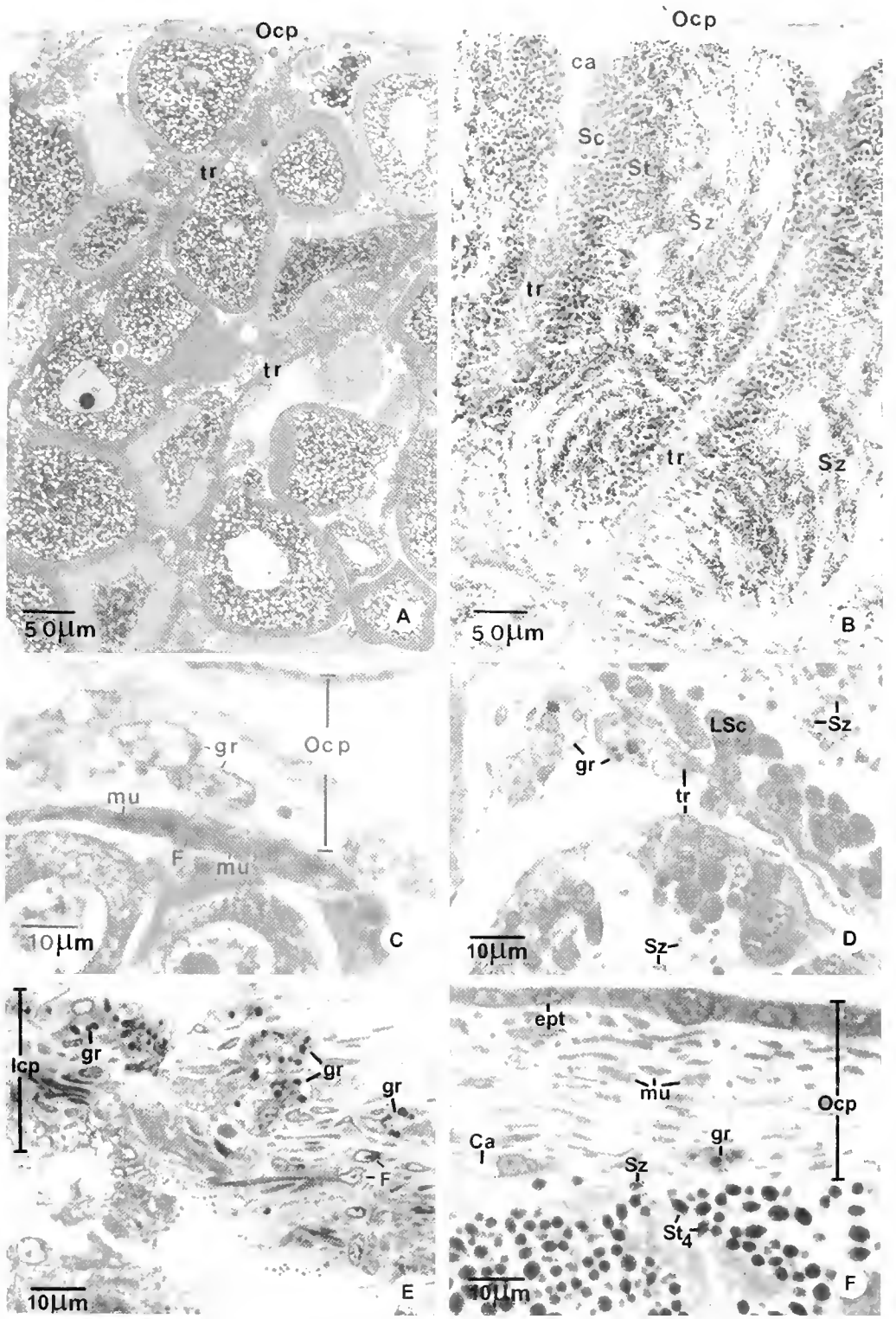

Figure 1. (A, B) Light micrographs of PAS-hematoxylin stained paraffin sections of *H. asinina* ovary (A) and testis (B) in the mature (prespawning) phase, where there are abundant mature stage V oocytes (Oc_5) with fully formed jelly coats (jc) in the ovary (in A), and numerous spermatozoa (S_z) with dense nuclei in the testis (in B). The connective tissue scaffolds of the gonads in both sexes consist of the outer capsule (Ocp), the trabeculae (tr) and the inner capsule (not shown). In the core of each trabecula, there is a small hemolymph capillary (Ca). Sc = spermatocytes, St = spermatids. (C, D) High magnification micrographs showing the granulated cells containing PAS positive granules (gr-in C) in the outer capsule of the ovary (Ocp) and in a trabecula (tr) of the testis (gr-in D). F = fibroblasts, LSc = leptotene spermatocyte, mu = muscle cells, Sz = spermatozoa. (E, F) The semi-thin plastic sections of the ovarian inner capsule (Icp- in E) and the testicular outer capsule (Ocp-in F) showing the granulated cells with their ellipsoid nuclei (gr-in E), and their attenuated processes containing dense granules (gr-in F). Ca = haemolymph capillary, ept = epithelium, F = fibroblast, mu = muscle cells, St_4 = spermatid stage 4.

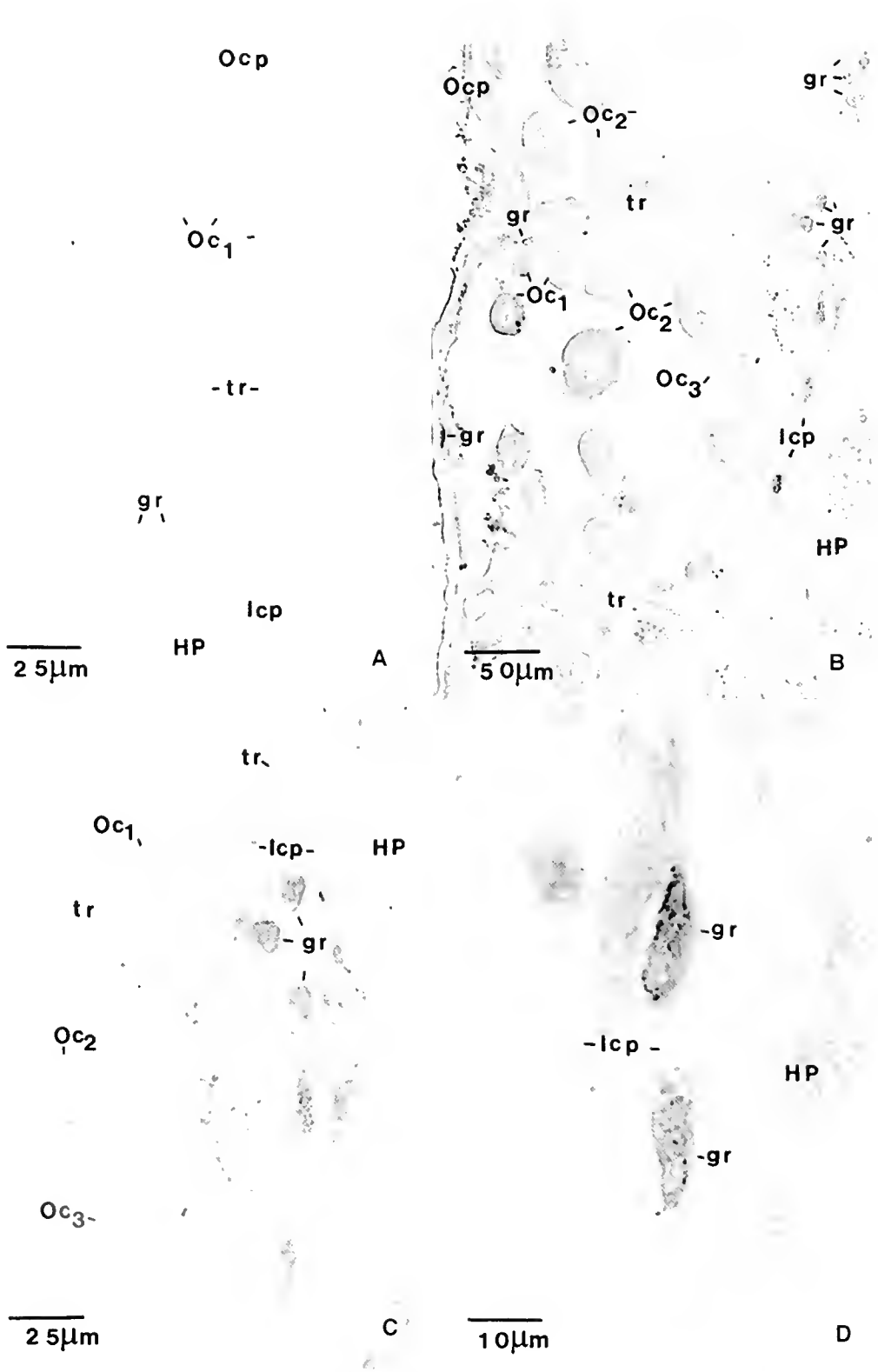

Figure 2. Light micrographs of the ovary in the proliferate phase, stained with anti-aELH by the immunoperoxidase method. (A) Control section shows no staining in the ovarian tissue. (B–D) Sections stained with anti-aELH, showing intense staining in granulated cells (gr) within the inner (Icp) and the outer capsules (Ocp), while moderate staining is seen in the connective tissue proper of trabecula (tr), both capsules (Ocp & Icp), and the cytoplasm of immature oocytes (Oc_1, Oc_2, Oc_3). HP = hepatopancreas.

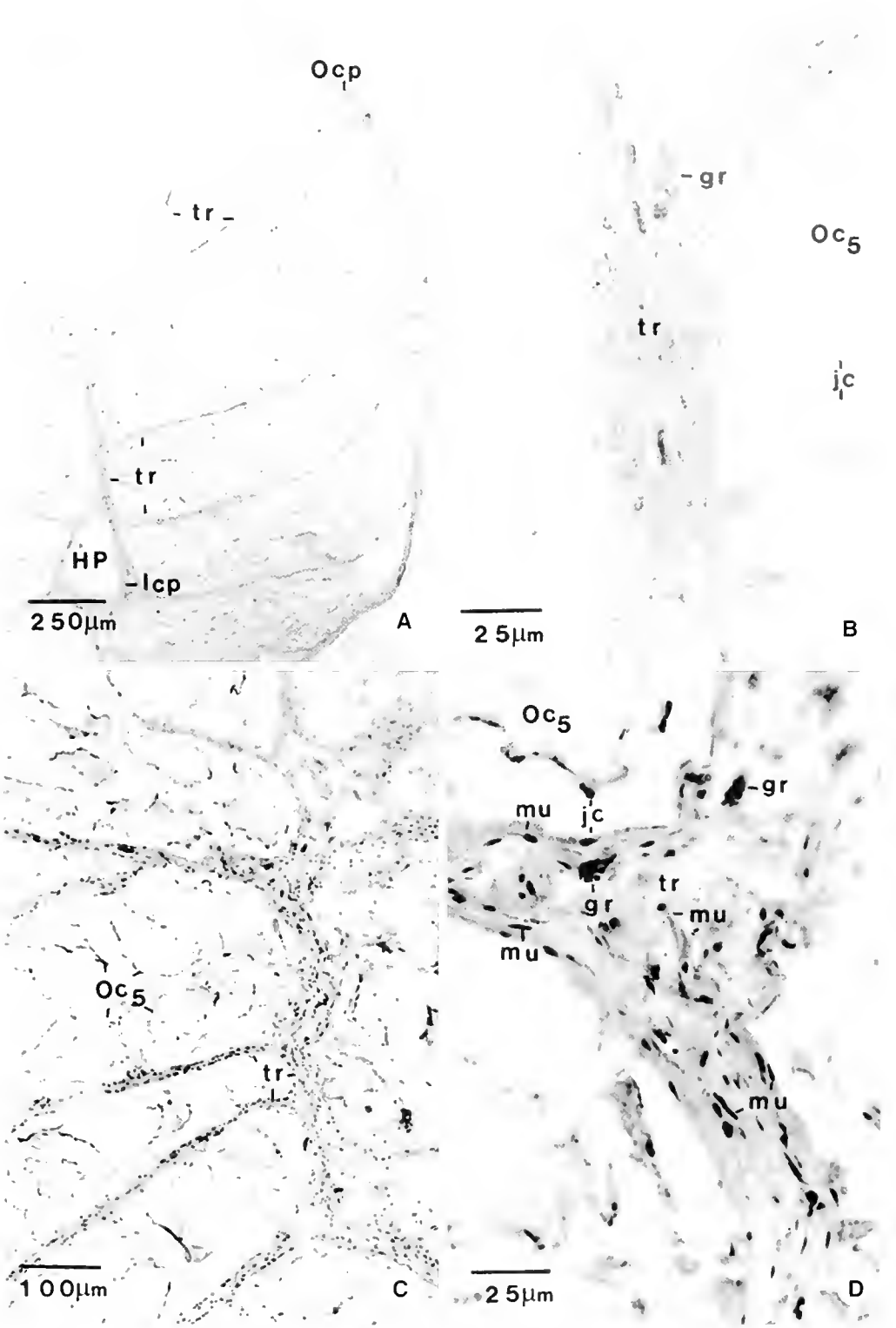

Figure 3. Light micrographs of the ovary in the mature phase, stained with anti-aELH by the immunoperoxidase method. (A, B) In A, anti-aELH exhibits staining in the trabeculae (tr) and both capsules (Ocp, lcp) of the gonad. In B, granulated cells (gr) in a trabecula are intensely stained. The cytoplasm of late stage oocytes (Oc_5) surrounded by a thick jelly coat (jc) is not stained. (C, D) Sections stained with anti-aELH and counter-stained with hematoxylin. In C, the connective tissue of the trabeculae (tr) is positively stained in comparison to oocytes (Oc_5). In D, which is higher magnification of an area from the trabeculae in C, granulated cells (gr) and muscle cells (mu) are intensely stained.

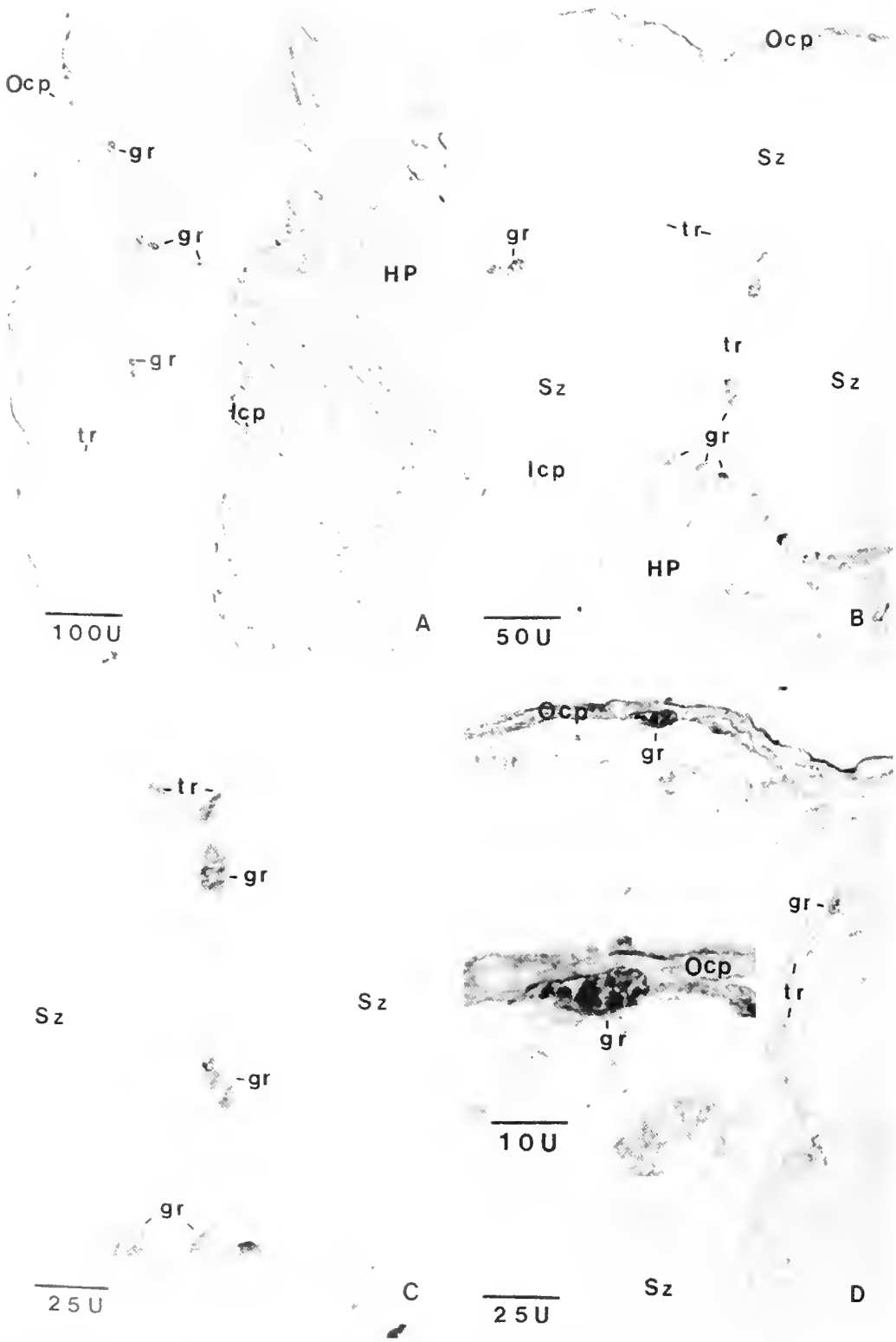

Figure 4. Light micrographs of the testis in the mature phase, stained with anti-aELH by the immunoperoxidase method. (A–D) Sections stained with anti-aELH, showing strong staining of the trabeculae (tr), both the outer and inner capsules (Ocp, Icp). In C, and D inset, which are high magnifications of a trabecula and the outer capsule, granulated cells (gr) with large granules in their cytoplasm are intensely stained, while spermatocytes and spermatozoa (Sz) are not stained.

is transported through the axon and released by exocytosis to the hemolymph (Dirks et al. 1989, Dirks et al. 1993, Van Minnen et al. 1988). Expression of CDCH was not restricted to the CNS alone, but was also found in the reproductive tract, including the oothecal gland, muciparous gland, and pars contorta, which are female accessory sex glands in *Lymnaea stagnalis*. In these glands the processes of positively labeled neurons terminated on the secretory cells, suggesting that they controlled the activities of these tissues (Van Minnen et al. 1988). CDCH immunoreactive material has also been found in secretory cells of the prostate gland and sperm duct (Van Minnen et al. 1989). In contrast to CDCH little is known about the origin of egg-laying hormone in abalone. Histological studies in the Japanese abalone, *Haliotis discus hannai*, showed that the number of neurosecretory cells, especially type 1 and 7, in pleural-pedal ganglia was correlated with the induction of spawning (Hahn 1992). Injections of pleural-pedal and visceral ganglion crude homogenates, or the combination of both, caused female *H. discus hannai* to spawn (Yahata 1973). The quantity of eggs being spawned was significantly greater with the injections of homogenates from visceral ganglion or the combination of pleural-pedal and visceral ganglion, when compared with the injection of pleural-pedal ganglion alone (Yahata 1973). In our preliminary study of *H. asinina* visceral ganglia, neurosecretory cells type 1 were also positively stained with anti-aELH (unpublished observation). Hence, existing evidence implies that abalone egg-laying hormone is mostly produced by neurosecretory cells of the nerve ganglia, particularly pleuropedal and visceral ganglia.

In the present study, we found that anti-aELH from *H. rubra* showed strong cross reaction with *H. asinina* gonadal connective tissues, and this implied that aELH may also be produced and stored in the granulated cells within the trabeculae and capsules of the gonad. Similarly, muscle cells in these connective tissue scaffolds were also stained with the anti-aELH, which suggested that this group of cells also bind aELH. Coggeshall (1972) suggested that, in *Aplysia*, ELH acted directly on muscle cells to induce their contraction, which caused the expulsion of ripe oocytes from the ovary. From the evidence gathered in the present study we, therefore, would like to suggest that the granulated cells in the trabeculae and the capsules of gonads in both sexes of abalone can synthesize aELH. After being released from the granulated cells, this hormone could bind to muscle cells in trabeculae and capsules and cause them to contract, which results in the expelling of ripe oocytes or spermatozoa from the gonads. The significance of the binding of anti-aELH to early stage oocytes is not known, but this hormone may also participate in the developmental process of germ cells. In contrast aELH did not bind to male germ cells thus its role in the male abalone may be limited to controlling the release of spermatozoa as has recently been demonstrated by our group (unpublished observation).

ACKNOWLEDGMENTS

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ULTRASTRUCTURE OF NEUROSECRETORY CELLS IN THE CEREBRAL AND PLEUROPEDAL GANGLIA OF *HALIOTIS ASININA* LINNAEUS

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ABSTRACT The ultrastructure of all three types of neurosecretory cells (NS_1, NS_2 and NS_3) in the cerebral and pleuropedal ganglia of *Haliotis asinina* was studied. NS_1 cells contained a euchromatic nucleus, and the cytoplasm contained RER, Golgi complexes, mitochondria and ribosomes. There were two types of neurosecretory granules in the NS_1 of cerebral ganglia, type 1 were large osmiophilic membrane-bound granules and type 2 were small electron-dense spherical granules. The NS_1 cells of pleuropedal ganglia only had one type of round cytoplasmic granule with a moderate to strong electron-dense matrix. NS_2 cells contained blocks of heterochromatin in the nucleus. The cytoplasm of the NS_2 of cerebral ganglia contained the usual organelles similar to those of NS_1 cells and large membrane-bound granules containing crystalline structures embedded in a moderately dense osmiophilic matrix. The NS_2 cells of pleuropedal ganglia contained one type of granule that had a dense matrix. NS_3 cells were smaller than NS_1 and NS_2. The nucleus contained thick heterochromatin strands. The organelles in the cytoplasm appeared to be fewer than those of NS_1 and NS_2. The secretory granules of NS_3 of both cerebral and pleuropedal ganglia were composed of aggregates of dense osmiophilic globules of various sizes.

KEY WORDS: *Haliotis asinina*, neurosecretory cells, cerebral ganglia, pleuropedal ganglia, ultrastructure

INTRODUCTION

Neurosecretory cells present in the cerebral ganglia of prosobranchs have not been extensively studied, and consequently little is known about them. The neurosecretory cells in the cerebral ganglia of *Bithynia tentaculata* Linnaeus were stained with phloxine (Andrews 1968). They were found to be unipolar and their nuclei were usually concave on one side. Neurosecretory material accumulated in the periphery of the cytoplasm and the axon hillock (Andrews 1968). In *Haliotis discus hannai* Ino and *Nordotis discus* Reeve, two cell types in the cerebral ganglia were identified as neurosecretory cells. They were large and medium sized cells with euchromatic nuclei and contained neurosecretory granules in the cytoplasm (Yahata 1971, Hahn 1994).

More recently, in the cerebral ganglia of *Haliotis asinina* Linnaeus, two types of neurosecretory cells were found (Upatham et al. 1997). These cells are either large or medium in size and stained positively with chrome-hematoxylin-phloxine and paraldehyde-fuchsin. The large sized cells contain a round nucleus with euchromatin and a distinct nucleolus. The medium sized cells also contain a round nucleus with patches of heterochromatin. Neurosecretory granules are present in both cell types (Upatham et al. 1997).

Most studies on the ultrastructure of neurosecretory cells in gastropods have concentrated on pulmonates and opisthobranchs with only a few on prosobranchs. In the cerebral ganglia of *Lymnaea stagnalis* (Linnaeus), two groups of neurosecretory cells have been described (Joosse 1964, Boer 1965, Boer et al. 1968). The cytoplasm of these cells contain electron-dense granules, which had a mean diameter of 20 nm, extremely elongated mitochondria, rough endoplasmic reticulum, free ribosomes, polyribosomes, Golgi complexes, multivesicular bodies, neurotubules and cytosomes. Bonga (1970), using the alcian blue-alcian yellow staining technique, reported that there was only one type of neurosecretory cells *i.e.* dark green cells in the pleuropedal ganglia of *L. stagnalis*. At the electron microscopic level, the dark green cells appear to contain a large quantity of elementary granules with a mean diameter of 20 nm. Numerous Golgi complexes were found to be evenly distributed in the cytoplasm, and there was extensive rough endoplasmic reticulum. A low number of cytosomes were present. In *Achatina fulica* (Bowdich), neurosecretory cells in the cerebral ganglia contain a round shaped nucleus with patches of heterochromatin and a conspicuous single large vacuole in the cytoplasm. In addition, electron-dense granules with a mean diameter of 16nm are associated with extensive Golgi complexes and rough endoplasmic reticulum (Kruatrachue et al. 1994).

In the prosobranchs, the ultrastructure of neurosecretory cells and neurons has been described in *B. tentaculata* (Andrews 1971) and *Haliotis rufescens* Swainson (Miller et al. 1973). In *B. tentaculata*, there are three types of neurosecretory cells, viz. S1, S2 and S3. In the cytoplasm of these cells, there are well-developed rough endoplasmic reticulum and Golgi complexes, mitochondria, lysosomes, glycogen granules, neurofibrils, and neurosecretory granules (Andrews 1971). Miller et al. (1973) describe that most of the neurons of *H. rufescens* contain the usual cytoplasmic organelles along with large membrane-bound inclusions. A few neurons contain small dense granules, which are similar in appearance to typical neurosecretory granules.

It was, therefore, apparent that an extensive investigation of the ultrastructure of neurosecretory cells in *Haliotis* was needed. Hence, the aim of the present study was to describe the ultrastructure of different types of neurosecretory cells in the cerebral and pleuropedal ganglia of *H. asinina*.

MATERIALS AND METHODS

Cerebral and pleuropedal ganglia from mature *H. asinina* were fixed in a mixture of 4% v/v glutaraldehyde and 2% v/v paraform-

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aldehyde in 0.1M Millonig buffer (pH 7.8) at 4°C for 24 h, washed several times with the same buffer, and postfixed in 1% OsO_4 in 0.1M Millonig buffer. The specimens were dehydrated in a graded series of ethanol, infiltrated in acetone and embedded in Araldite 502-epoxy resin. Sections were cut on a Sorvall MT2 ultramicrotome, stained with saturated uranyl acetate and lead citrate, and viewed with a Hitachi H-300 TEM, operating at 75KV.

RESULTS

Based on ultrastructural characteristics, there are three types of neurosecretory cells in the cerebral and pleuropedal ganglia of *H. asinina*. These are: type 1 neurosecretory cell (NS_1), type 2 neurosecretory cell (NS_2), and type 3 neurosecretory cell (NS_3).

NS_1 cells are round to oval (15–20 μm in dimension) and contain round nuclei (6–8 μm in diameter) (Fig. 1, Fig. 2). The

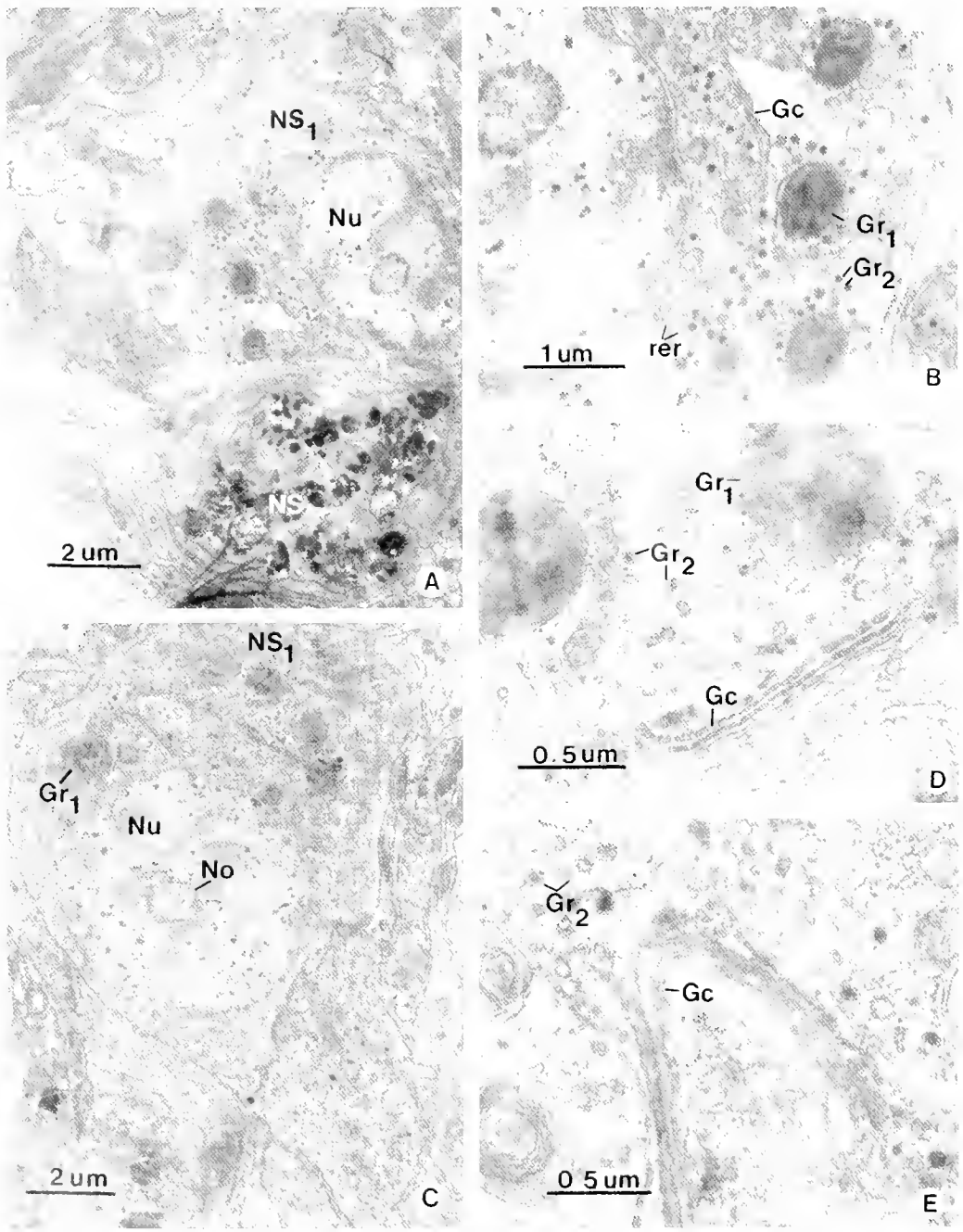

Figure 1. TEM micrographs of NS_1 cells of cerebral ganglia. (A, C) Medium power micrographs of the NS_1 showing the round nucleus (Nu) which contains a thin rim of heterochromatin (He) near the nuclear envelope. The nucleolus (No) is round, large and very distinct. There are abundant secretory granules in the cytoplasm. NS_1, type 1 neurosecretory cell; NS_3, type 3 neurosecretory cell; Gc, Golgi complex; Gr_1, type 1 granule. (B, D, E) High magnifications of the cytoplasm of the NS_1 demonstrating abundant rough endoplasmic reticulum (rer), Golgi complexes (Gc) and secretory granules. Type 1 granules (Gr_1) are large spherical membrane bound granules whereas type 2 granules (Gr_2) are small and round, containing electron-dense cores.

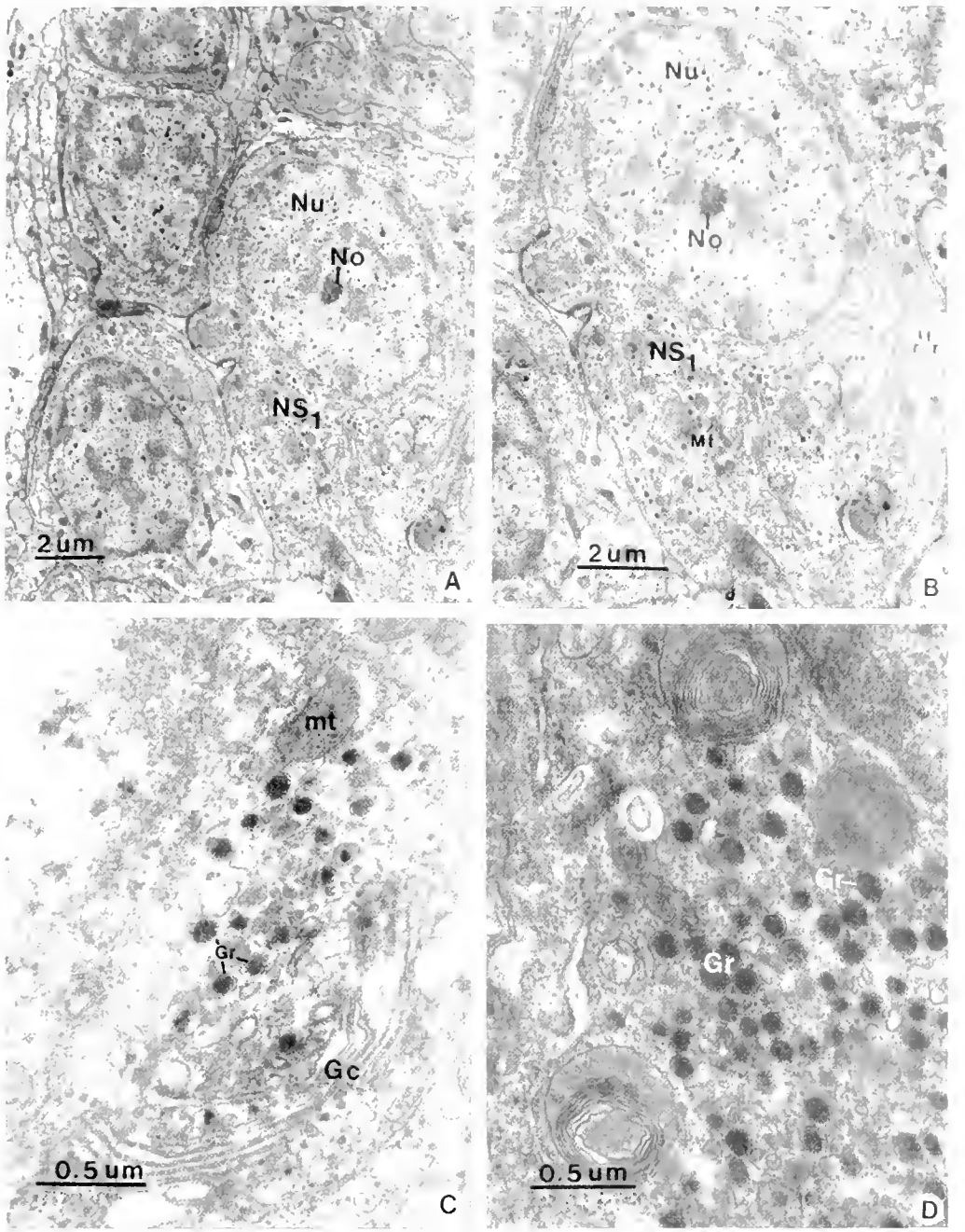

Figure 2. TEM micrographs of NS_1 cells of pleuropedal ganglia. (A, B) Low and medium power micrographs of NS_1 showing a round nucleus (Nu) which contains a thin rim of heterochromatin (He) near the nuclear envelope. The nucleolus (No) is round and very distinct. The cytoplasm contains rough endoplasmic reticulum (rer), mitochondria (Mt), Golgi complexes (Gc) and secretory granules (Gr). (C, D) Enlarged view of A & B exhibiting the cytoplasm of NS_1 containing mitochondria (Mt), extensive Golgi complexes (Gc) and electron-dense granules (Gr) near the maturing face of the Golgi complexes.

nucleus contains a thin rim of heterochromatin near the nuclear envelope. Small patches of heterochromatin are scattered in the central region of the nucleus, while the rest of the nucleoplasm contains finely dispersed euchromatin. The nucleolus is large, round and very prominent (Figs. 1A, C, Figs. 2A, B). The cytoplasm shows abundant rough endoplasmic reticulum consisting of many large stacks of membrane in straight arrays (Fig. 1D, Fig. 2C). Golgi complexes are extensively developed and each consists of four to five elongated and slightly bent cisternae and saccules, which are often surrounded by dense vesicles (Fig. 1D, Fig. 2C). There are mitochondria and abundant polyribosomes in the cytoplasm. Two types of secretory granules are present in the NS_1 of cerebral ganglia. Type 1 are large, membrane-bound, spherical granules (Figs. 1B, C). Their diameter is about 500–800 nm and they contain moderately osmiophilic material. There are few granules present, and these are usually dispersed around the maturing face of the Golgi complexes (Fig. 1D). Type 2 are small membrane-bound spherical secretory granules, containing electron-

dense matrices. Their diameter is about 60 nm, and they are scattered throughout the cytoplasm (Figs. 1B, D). The newly synthesized granules indicated by their light matrices are concentrated mainly in the maturing face of the Golgi complexes (Figs. 1D, E). In the NS_1 of pleuropedal ganglia, only one type of secretory granule is present. These granules are round and have a mean diameter of 120 nm. They contain moderate to strong electron-dense matrices (Figs. 2C, D).

NS_2 cells are round or oval (10–12 μm in dimension) (Fig. 3, Fig. 4). Their nucleus is round (6–8 μm in diameter) with a thin rim of heterochromatin near the nuclear envelope, and large blocks of heterochromatin are scattered in the central region (Fig. 3A, Fig.

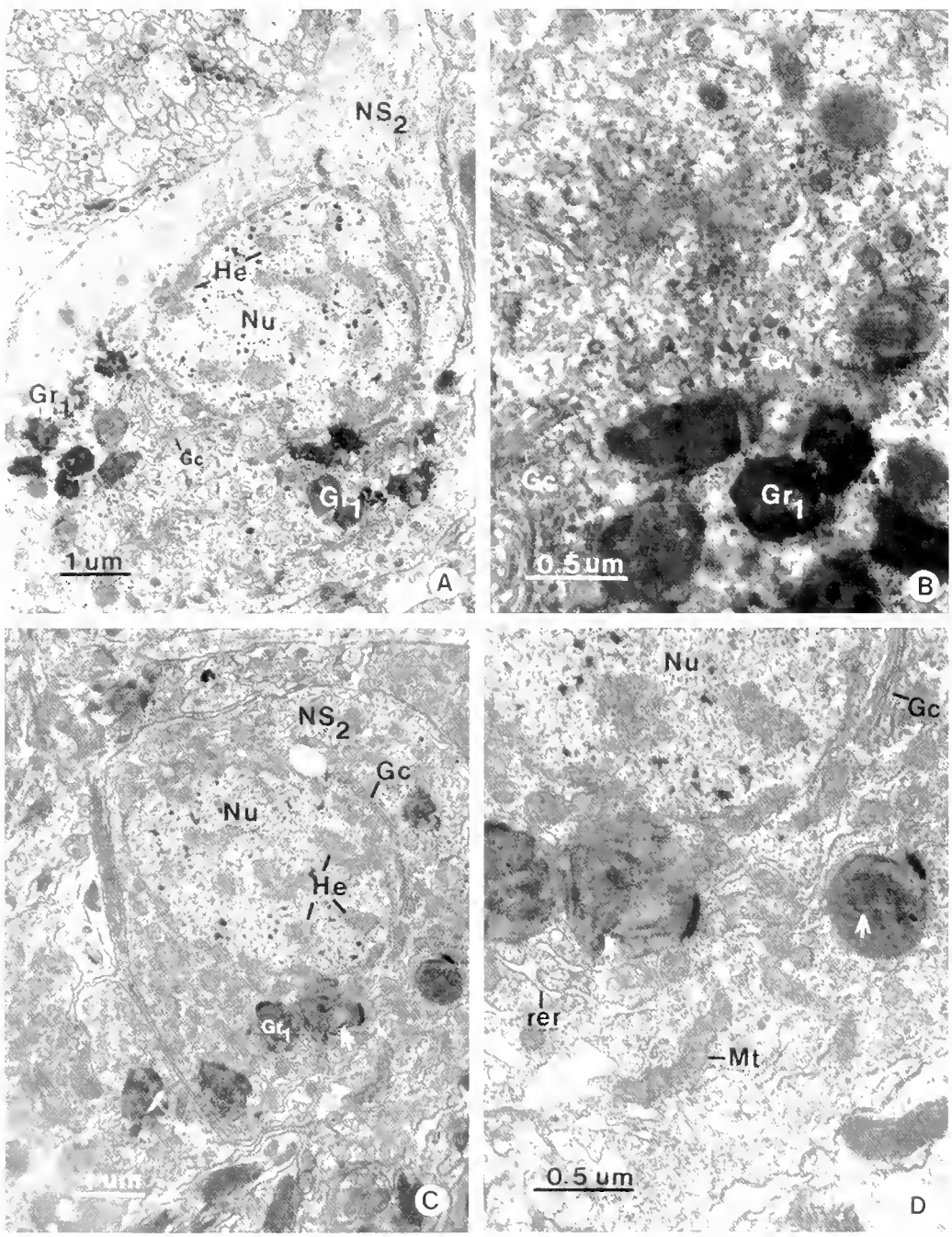

Figure 3. TEM micrographs of NS_2 cells of cerebral ganglia. (A, C) Medium power micrographs of NS_2, exhibiting the round nucleus (Nu) with a thin rim of heterochromatin (He) attached to the nuclear envelope and large blocks of heterochramatin scattered in the central area of the nucleus. The cytoplasm possesses rough endoplasmic reticulum, mitochondria, Golgi complexes (Gc), and type 1 granules (Gr_1) containing a crystalline structure (arrow). (B, D) Enlarged view of C exhibiting the cytoplasm of the NS_2 containing Golgi complexes (Gc), rough endoplasmic reticulum (rer), mitochondria (Mt), condensing vesicles (Cv) and secretory granules. Type 1 granules (Gr_1) contain a crystalline structure (arrow) embedded in a moderately osmiophilic matrix. Nu, nucleus.

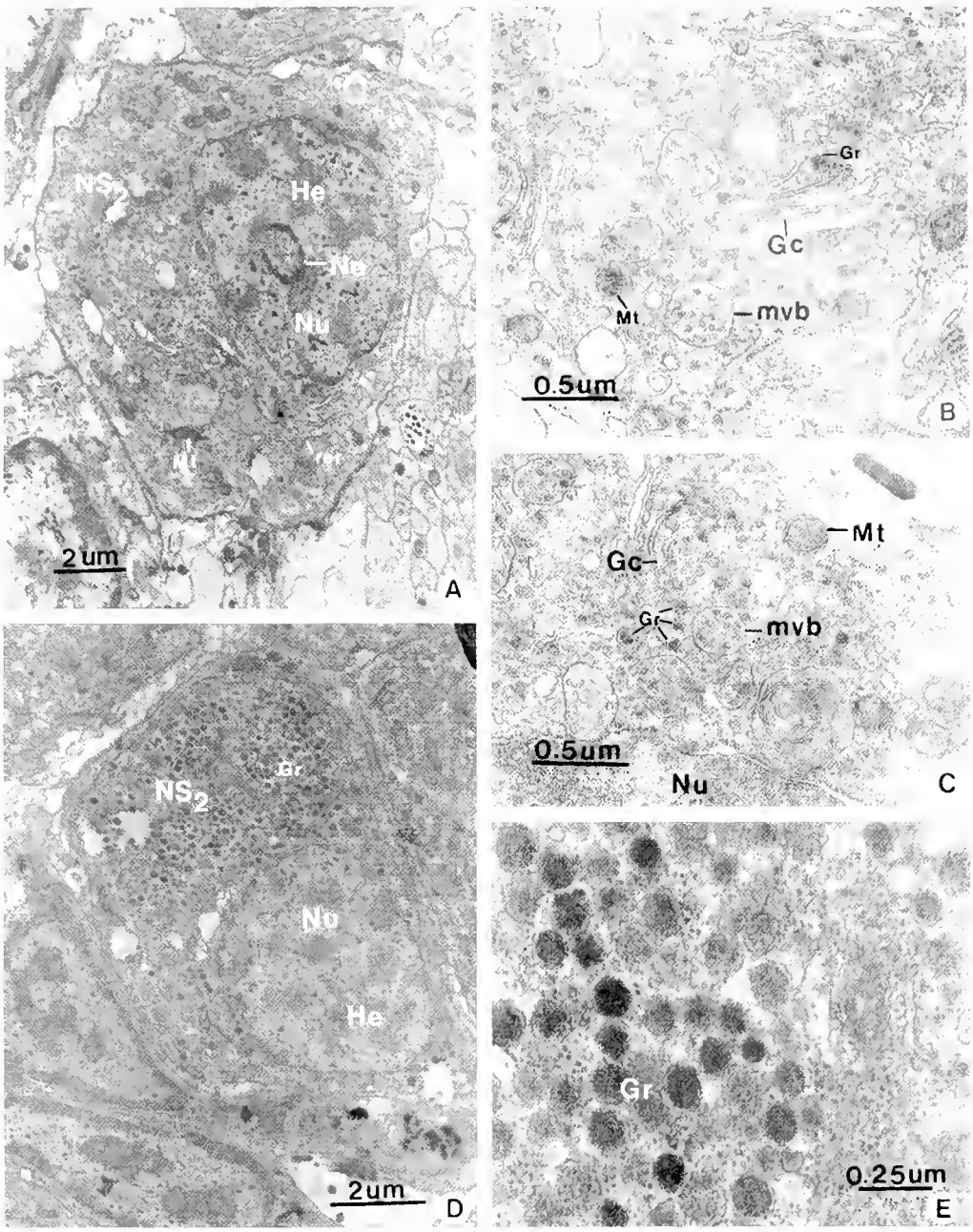

Figure 4. TEM micrographs of NS_2 cells of pleuropedal ganglia. (A) An electron micrograph of subtype a of NS_2 showing round nucleus (Nu) with a thin rim of heterochromatin (He) along the periphery and in the central region of the nucleus. The nucleolus (No) is very prominent. Mt, mitochondria; rer, rough endoplasmic reticulum. (B, C) Enlarged view of A, exhibiting the cytoplasm of NS_2 containing Golgi complexes (Gc), mitochondria (Mt), multivesicular bodies (mvb) and secretory granules (Gr). Nu, nucleus. (D) An electron micrograph of subtype b of NS_2, exhibiting a cytoplasm filled with a large number of spherical secretory granules (Gr). Nu, nucleus; He, heterochromatin. (E) An enlarged view of the spherical secretory granules (Gr) with dense matrices.

4A). The cytoplasm of the NS_2 cells in the cerebral ganglia possesses extensively developed rough endoplasmic reticulum, and has numerous mitochondria (Fig. 3D). There could be more than one Golgi complex per cell. Each Golgi complex usually consists of four to six elongated cisternae and saccules, which are often surrounded by dense vesicles (Fig. 3B). The most prominent granules appear large and round, membrane-bound, and about 500 to 800 nm in diameter. Each granule contains crystalline structures of various sizes, embedded in moderately dense osmiophilic matrices (Figs. 3B, D). These granules are concentrated at the maturing face of Golgi complexes (Fig. 3D).

The NS_2 cells of the pleuropedal ganglia contain abundant cell organelles, i.e., mitochondria, rough endoplasmic reticulum, Golgi complexes, multivesicular bodies (Figs. 4B, C), and small spherical secretory granules with dense matrices about 240 nm in diameter (Figs. 4D, E). NS_2 cells can be divided into two subtypes, viz subtypes A and B. Subtype A contains few neurosecretory granules, which are mostly concentrated at the Golgi complexes, and

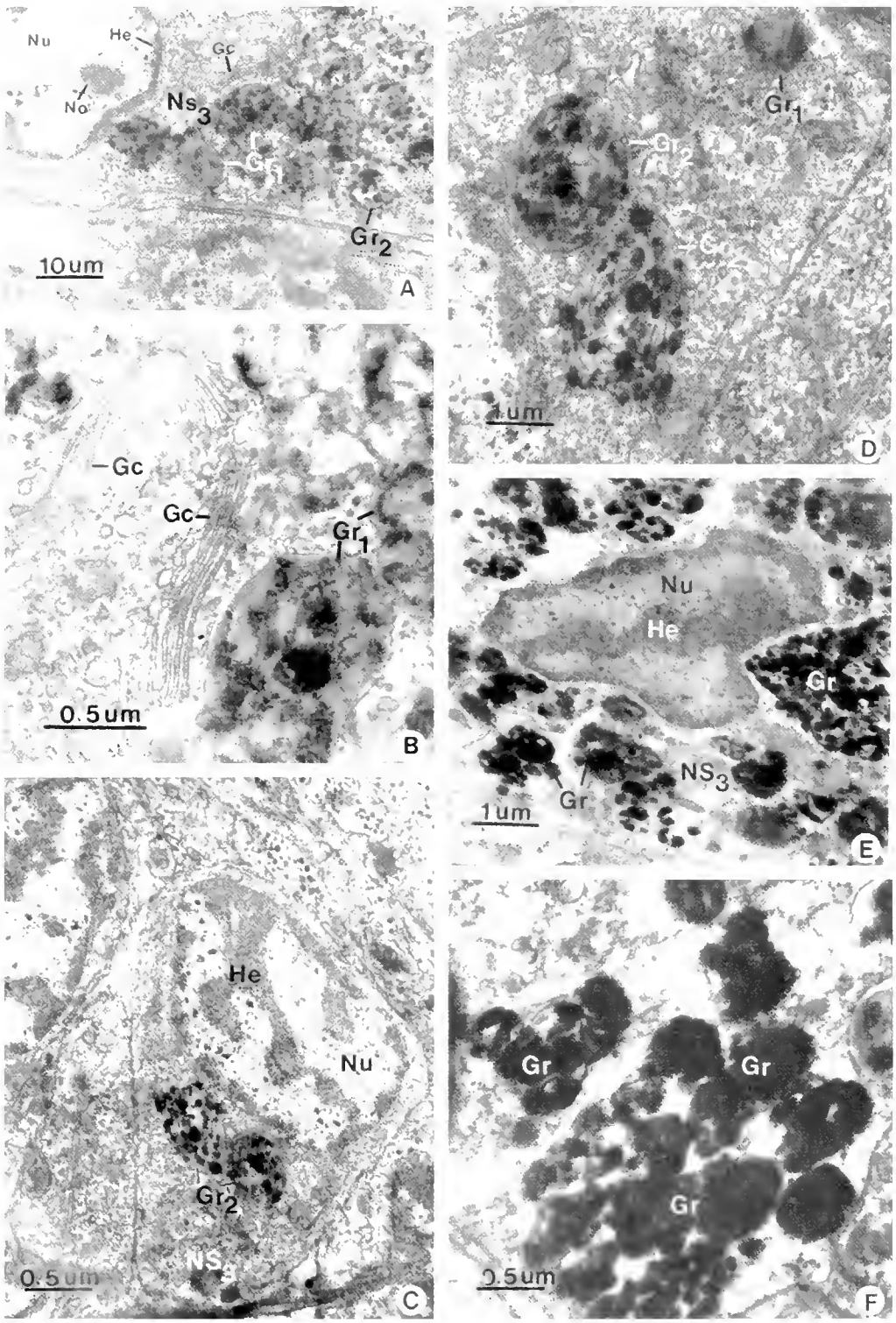

Figure 5. TEM micrographs of NS_3 cells of cerebral ganglia. (A) Medium power micrograph of NS_3 showing an oval nucleus (Nu) which contains thick strands of heterochromatin (He) near the nuclear envelope. The nucleolus (No) is very distinct. The cytoplasm contains rough endoplasmic reticulum, mitochondria, Golgi complexes (Gc) and secretory granules. Type 1 granules (Gr_1) are composed of strong osmiophilic material. Type 2 granules (Gr_2) are composed of highly condensed osmiophilic globules. (B) A high magnification of the cytoplasm of NS_3 showing extensive Golgi complexes (Gc) and type 1 granules (Gr_1). (C) Medium power micrograph of NS_3 showing an oval nucleus (Nu) containing thick strands of heterochromatin (He) near the nuclear envelope and blocks of heterochromatin passing through the center of the nucleus. (D) A high magnification of the cytoplasm of NS_3 containing type 1 granules (Gr_1) and type 2 granules (Gr_2). (E) Medium power micrograph of NS_3 showing an indented nucleus (Nu) which contains thick strands of heterochromatin (He) near the nuclear envelope and blocks of heterochromatin passing through the center of the nucleus. The cytoplasm contains aggregated granules (Gr). (F) An enlarged view of E exhibiting aggregated granules (Gr).

there is a large amount of rough endoplasmic reticulum, numerous mitochondria and extensively-developed Golgi complexes. Hence, they show evidence for high secretory activity (Figs. 4A, B). In contrast, cytoplasm of subtype B is filled mostly with neurosecretory granules and has few additional organelles (Fig. 4D). These cells appear to be in a storage phase where granules are ready to be discharged.

NS_3 cells are smaller (8–10 μm in size) than NS_1 and NS_2 (Fig. 5, Fig. 6). The nuclei are oval (about 5–6 μm in dimension) and some are indented (Figs. 5C, E, Figs. 6A, C, D). They contain a

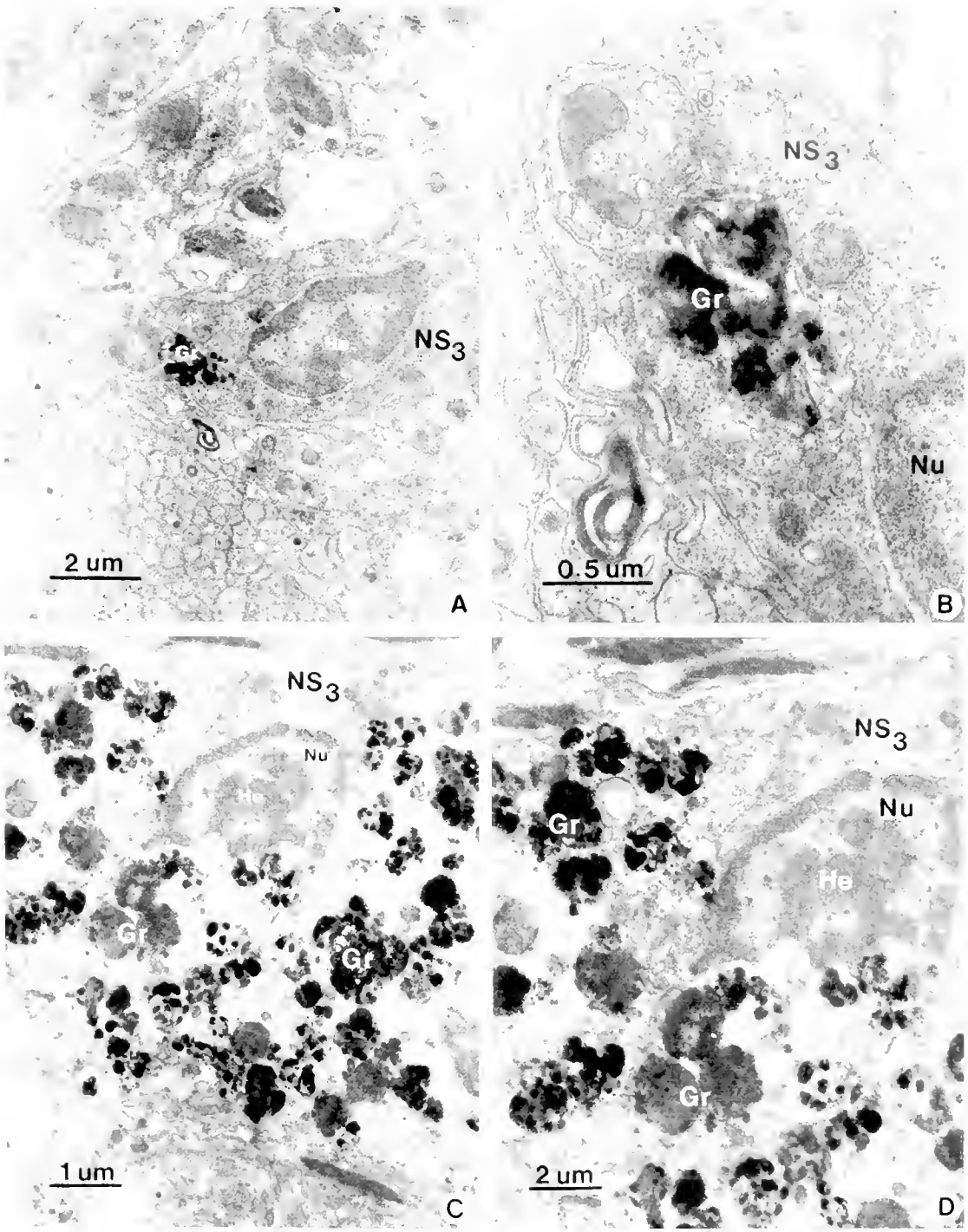

Figure 6. TEM micrographs of NS_3 cells of the pleuropedal ganglia. (A, B) Low and high power micrographs of NS_3 subtype 1 showing oval nuclei (Nu) which contain thick strands of heterochromatin (He) at the nuclear envelope and in the central region. The cytoplasm contains few large secretory granules (Gr). (C, D) Low and high power micrographs of NS_3 subtype 2, showing an oval nucleus (Nu) which contains thick strands of heterochromatin (He) near the nuclear envelope and in the central region. The cytoplasm contains aggregated granules (Gr).

thick rim of heterochromatin near the nuclear envelope, and a thick strand of heterochromatin in the center (Figs. 5C, E, Fig. 6D). In the NS_3 cells of the cerebral ganglia, the cytoplasm contains rough endoplasmic reticulum, mitochondria, and Golgi complexes, but these organelles are less abundant, and of smaller sizes, than those in NS_1 and NS_2 cells. Much cytoplasm is filled with secretory granules (Figs. 5A, D). These granules are large (2000–7000 nm in diameter), membrane-bound, and contain dense, osmiophilic globules of various sizes that are aggregated together. When examined in detail, these granules are divided into two subtypes. Type 1 secretory granules are composed of strongly osmiophilic crystalline material in a clear ground substance (Figs. 5A, B), and type 2 secretory granules are composed of highly condensed osmiophilic globules aggregated together (Figs. 5D, E, F). It is possible that type 2 secretory granules are developed from type 1 secretory granules.

In the NS_3 cells of pleuropedal ganglia, the cytoplasm contains less rough endoplasmic reticulum, fewer mitochondria, and fewer Golgi complexes, than those of NS_1 and NS_2. NS_3 cells are divided into two subtypes. Subtype 1 cytoplasm contains few, but large secretory granules, with a strong osmiophilic substance within a clear ground matrix (Figs. 6A, B), and subtype 2 cytoplasm contains numerous large secretory granules, each composed of strong osmiophilic globules aggregated together in a homogeneous ground substance (Figs. 6C, D). It is possible that neurosecretory cells of subtype 1 develop from subtype 2 through the condensation and dehydration of the osmiophilic substance.

DISCUSSION

The ultrastructural study of the neurosecretory cells in the cerebral ganglia of *H. asinina* revealed that there are three types of neurosecretory cells (i.e. NS_1, NS_2, NS_3). This is in contrast to only two types reported by Upatham et al. (1997) using light microscopy. The pleuropedal ganglia also contain three types of neurosecretory cells. The details of the heterochromatin and euchromatin in the nuclei of cerebral and pleuropedal ganglia neurosecretory cells were revealed by light microscopy and confirmed by the extra resolution of TEM. The NS_1 nucleus contains mostly euchromatin, while large amounts of heterochromatin were present in NS_2 and NS_3 cells. In general, the cytoplasm of these cells resembles that of neurosecretory cells described in other gastropods, such as *B. tentaculata* (Andrews 1971), *H. rufescens* (Miller et al. 1973), *A. fulica* (Kruatrachue et al. 1994) and *L. stagnalis* (Boer et al. 1968).

The main differences between the neurosecretory cells of the cerebral ganglion and those of the pleuropedal ganglion are the type and size of neurosecretory granules. In the cerebral ganglia, the NS_1 cell contains 2 types of secretory granules (large and small) while the NS_1 cell of the pleuropedal ganglion contains only one type (small granules). In addition, the NS_2 cells of the cerebral and pleuropedal ganglia both contain one type of secretory granule. However, they are different both in size and content. The NS_3 of both ganglia appear to contain one type of secretory granule that contains aggregates of dense globules.

In the cerebral ganglia, the NS_1 cytoplasm is composed of cell organelles, including numerous mitochondria, rough endoplasmic reticulum, Golgi complexes and secretory granules, which reflects a highly active secretory function. Golgi complexes are extremely large and there may be several present in a cell. Small electron-dense secretory granules are associated with the maturing face of Golgi complexes. These granules were later widely distributed throughout the cytoplasm. Hence, this indicates that the Golgi complexes in the neurosecretory cells of *H. asinina* have a similar role in packing of electron-dense material, to those of neurosecretory cells reported in other gastropods (Boer et al. 1968, Kai-Kai & Kerkut 1979).

The ultrastructural characteristics of the NS_1 of the cerebral ganglia indicate that it is a highly active synthetic cell. In comparison, the cytoplasm of NS_2 and NS_3 contains only one type of granule, which are large and round in NS_2 and polymorphic in NS_3. These granules bear crystalline structures in NS_2 and in NS_3 and are composed of aggregates of dense osmiophilic substances. The similarity between granules in NS_2 and NS_3 tends to indicate that the two cells could be of the same group. While the NS_2 appears to be in a more active secretory phase, the NS_3 has reached the fully differentiated state, in which hormonal product is already produced in abundance, stored, and ready for release.

In the present study, the cytoplasms of NS_1 and NS_2 cells in the pleuropedal ganglia of *H. asinina* exhibit characteristics which imply that they have actively synthetic features. These are the numerous mitochondria, rough endoplasmic reticulum, Golgi complexes, multivesicular bodies and secretory granules, and numerous small clear vesicles that may be transport vesicles in the cytoplasm. Rough endoplasmic reticulum and Golgi complexes are well developed. The electron-dense secretory granules are probably formed from the Golgi complex. This process is similar to that described in neurosecretory cells in the pleural ganglia of *L. stagnalis* (Bonga 1970).

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INTERACTIONS AMONG RED ABALONES AND SEA URCHINS IN FISHED AND RESERVE SITES OF NORTHERN CALIFORNIA: IMPLICATIONS OF COMPETITION TO MANAGEMENT

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ABSTRACT Red abalones (*Haliotis rufescens*), red sea urchins (*Strongylocentrotus franciscanus*), and purple sea urchins (*S. purpuratus*) share similar food and habitat requirements in northern California. Red abalones and red sea urchins also support important fisheries. Here we explore spatial interactions and apparent competitive effects among these species at an area where fishing has large impacts on both taxa, and at unfished reserve sites in which invertebrate density and food availability differ. There was an inverse correlation between adult red abalone and red sea urchin abundance at the scale of our transects when density of either or both species was high. In the poorest habitat for macroalgae, red abalones seldom occurred on the same transects with red urchins. The results suggest that differences in density, depth, and food availability play an important role in the observed spatial patterns of red abalones and red sea urchins. Purple sea urchins were not correlated to either of the other two species' distributions. An intense fishery for red sea urchins appears to have had a positive effect on kelp availability, and abalone growth and abundance. Aerial photographs during the period of intense urchin fishing (from 1982 to 1989), showed a dramatic increase in the surface canopy. Similarly, during this period, size frequency distributions of fished red abalones show an increase in the number of individuals in larger size classes. Modal progression in abalone size frequency distributions suggests a faster growth rate during this period when compared with a growth study, at the same location, conducted during the pre-urchin fishery years. Ultimately, red sea urchin removal apparently led to an increase in red abalone abundance even at a site that was heavily fished by recreational abalone fishers. Meanwhile, at a nearby reserve site where kelp populations are lower, red abalones have declined in abundance as red sea urchins increased. Our results suggest the need for multi-species ecosystem-based approaches to management of these valuable resources.

KEY WORDS: reserves, *Haliotis rufescens*, *Strongylocentrotus franciscanus*, *Strongylocentrotus purpuratus*, competition, spatial exclusion, ecosystem-based management

INTRODUCTION

Red abalones (*Haliotis rufescens*), red sea urchins (*Strongylocentrotus franciscanus*) and purple sea urchins (*S. purpuratus*) share similar food and habitat preferences in kelp forest communities along the California coast. In northern California, red abalones are found in rocky intertidal and shallow subtidal areas in high abundance at 7–8-m depths, but also occur down to 25 m in areas where drift algae accumulate in surge channels. Red and purple urchins are found from mid- to low- intertidal zones to depths in excess of 50 m. Both species prefer rocky substrates, particularly ledges, crevices and surge channels, and avoid sand and mud (Schroeter 1978, Kato & Schroeter 1985). In areas of high predation, red abalones show a preference for crevice habitat (Hines & Pearse 1982). Red abalones and red and purple urchins feed primarily on the same species of macroalgae (Leighton & Boolootian 1963), and have been described as potential competitors for food and space (Leighton 1968, Tegner & Levin 1982). Both urchins and abalones feed primarily on drift kelp, but sea urchins are well known for their destructive grazing on attached plants when drift becomes limiting. Schroeter (1978) presented evidence that red urchins out compete purple urchins for food and habitat, suggesting that the red urchin fishery could lead to an increase in populations of its smaller congener. Several authors have considered the potential of sea urchin populations being released from competition as abalones were fished down in southern California (North & Pearse 1970, Tegner 1980, Tegner & Levin 1982, Tegner & Dayton 2000). No one has examined the effects of red urchin removal in areas where red abalones are still abundant.

Red abalones and red sea urchins are both fished intensively in northern California. Red abalone take is restricted to recreational fishers who are prohibited from using SCUBA. Karpov et al. (1998) reported that this results in a "defacto" refuge for red abalone at depths greater than 8.4 m. Since 1985, Red urchins have been subjected to an intense commercial fishery at all depths (Kalvass & Hendrix 1997). Purple sea urchins are essentially unfished, comprising less than 1% of the total urchin landings.

The red abalone, the largest member of the genus, attains sizes of up to 312 mm in northern California (Department of Fish and Game -DFG- unpublished data). Legal minimum size for the recreational-only abalone fishery is 178-mm shell diameter. Red sea urchins, characterized by long spines in relation to test diameter (TD), attain sizes of 140 mm TD, and were first protected by a minimum size limit of 89-mm TD in 1991. Purple sea urchins have short spines relative to their TD, and maximum TD is about 85 mm. In northern California macroalgae are highly seasonal as a food source (Tegner et al. 1992). Wave energy is markedly higher in northern compared to southern California, and thus is likely to be a more important factor affecting the distribution of abalone and urchins (Deacon 1973).

Fishing effects, both direct and indirect, make field studies of competition difficult to conduct. Here we take advantage of three locations in northern California, one fished and two reserves closed to sport and commercial fishing, to examine biological and fishery interactions between red abalones, red sea urchins, and purple sea urchins. Differences in fishing regimes constitute a natural experiment, which offers an opportunity to examine the factors structuring this nearshore community, the potential produc-

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tivity of the three grazers, and the ecosystem effects of fishing. We compare observations of densities, size-frequency distributions, and spatial distributions of the three grazers and kelp populations over time. Two major questions are asked in this study: (1) Has intense red urchin removal by the fishery had an affect on the abalone resource, and (2) are there significant differences by habitat, density, or depth that indicate spatial exclusion between sea urchins and abalones.

Materials and Methods

Our study is focused in northern California on a fished area, Van Damme State Park (VDSP), and two unfished "control" areas: Bodega Marine Life Refuge (BMLR) and Point Cabrillo Marine Reserve (PCMR)(Fig. 1). VDSP is highly impacted by both the sport only abalone fishery and the commercial red sea urchin fishery. In this study, we used the same 120 dive stations examined for changes in emergent abalone abundance and size distributions during 1986, and from 1989–1992 by Karpov et al. (1998). In 1999, we added 34 more stations that were at comparable locations to those surveyed during earlier years. Red urchins were surveyed throughout these periods; purple sea urchins were added in 1989. As in the previous study (Karpov et al. 1998), we stratified our sampling into two strata, "shallow" and "deep", using 8.4 m as the dividing depth because free divers seldom dive deeper than 8.4 m to collect abalone. Thus, the VDSP study area represents a treatment of large-scale continued removal of red urchins at all depths and red abalones from shallow water. Two no-take reserves were also surveyed, PCMR and BMLR (Fig. 1). Parker et al. (1988) first surveyed 30 stations at PCMR in 1986. PCMR was re-sampled in 1988 for sea urchins at 14 stations and again in 1999 at 30 stations. About half of the 1999 stations were in close proximity to locations sampled in 1986. Sampling was normally conducted during late summer months. Station depths throughout the study period ranged from 2 to 18 m. Sampling was conducted along 2 × 30 m transects randomly placed on rocky habitat. Transects were not sampled if they were placed on substrate with more than 50% sand.

PCMR, first established as a "no-take" reserve in 1975, is located 9 km north of VDSP. PCMR provides both a site for comparing spatial correlation for high densities of red and purple sea urchins to high densities of red abalone, as well as a nearby unfished control for comparison with the fished VDSP site. BMLR, located 130 km south of VDSP, was established as a no take reserve in 1966 (Fig. 1). In 1999, 33 stations at BMLR were sampled to examine spatial correlation in an area with no surface canopy of kelp in more marginal abalone and urchin habitat. We also examined this area on a smaller spatial scale. Stations were grouped into three sites of distinct habitat, designated Horseshoe Cove, Cave, and Points (North and South Point combined) (Fig. 2).

To compare the relative availability of food at each study area, percent cover of algae was estimated by divers on each quadrant and averaged in all three of the study sites during 1999. Algae and cover were classified in five categories: encrusting, coralline algae turf, foliose, understory, or canopy kelp. Understory kelp included brown algae such as *Pterygophera californica* and *Laminaria dentigera*. The primary canopy species in northern California was the annual kelp, *Nereocystis luetkeana*. Percentage cover at times exceeded 100%.

Size frequency distributions, weighted for density, were examined for patterns of recruitment of red sea urchins and red abalones at VDSP and PCMR. Size frequency distributions of red urchin catches were obtained from commercial samples. Estimates of

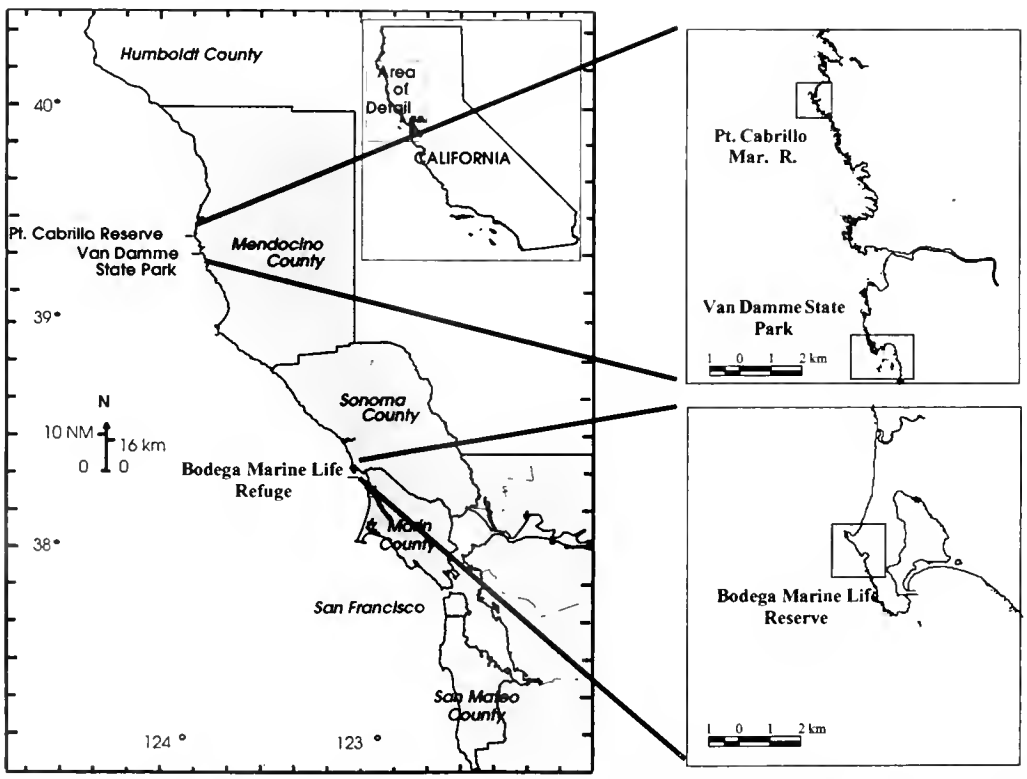

Figure 1. Location of Point Cabrillo Marine Reserve (PCMR), Van Damme State Park (VDSP), and Bodega Marine Life Refuge (BMLR) study areas in northern California.

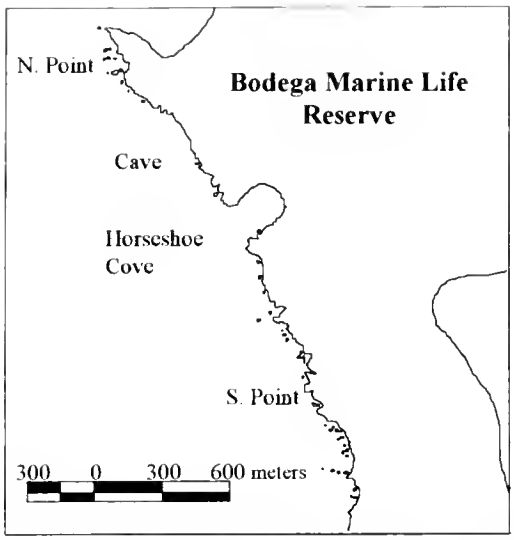

Figure 2. Detailed view of BMLR showing sampling areas.

urchin catch at VDSP were made from fishery logbook data. Creel surveys of sport-caught red abalones conducted during spring minus-tides from 1977 to 1994 at VDSP were used to construct size frequency distributions of the sport take.

Estimates of kelp surface canopy were obtained at VDSP from aerial infrared photographs taken each October from 1982 to 1991. Surface areas estimates were constructed from polygons drawn visually in Arc View GIS software.

Statistical Comparison

Red abalone and red sea urchin counts from 2 × 30 m transects at VDSP and PCMR were compared using two-way ANOVAs testing differences between year and depth strata. Densities were transformed using the method of Pearse and Hines (1987) (transformed density = ln (density + 1)). The non-parametric Spearman Rank Correlation test was used to examine inter-specific spatial correlation. Paired counts of each of the three species were used. The test produces a correlation coefficient (r_s) range 1–1. Transects with zero counts were excluded from these comparisons to avoid inappropriate habitat.

We used 1986 PCMR data to examine the effect of transect size on the observed inverse correlation between red abalones and red sea urchins. Since data that year were recorded on a finer scale, subdividing the 2 × 30 m transect into six 1 × 10 m segments, we examined spatial correlation by randomly selecting a 10, 20, and 60 m^2 segment from each of 26 shallow and deep transects. The inverse relation was significant at both 20 ($r_s = -0.55$, $P = 0.004$) and 60 m^2 ($r_s = -0.43$, $P = 0.03$) transect sizes, but not significant at 10m^2.

Red abalone and sea urchins were classified as having either low or high abundance at each of the three locations across the years examined, depth strata, and habitat types. This classification was used to create a matrix allowing the significance of the correlation coefficients related to high or low density of any of the species to be examined. The abundance of red abalone, red sea urchin, and purple sea urchin was considered high at greater than 0.4, 1.0, 1.0 m^{-2}, respectively. Similarly, inter-specific spatial correlation was examined at each of the three habitat types at BMLR and between the three study areas. Finally, correlation was also examined at VDSP and PCMR over time to determine if fishery related abundance changes reflected spatial exclusion. Significance for all tests was set at $\alpha = 0.05$.

RESULTS

During the 1999 survey, BMLR had the least macro-algae of the three study areas. While relative cover of foliose algae was comparable to the other two study areas, canopy was absent and understory much less abundant (Table 1). Horseshoe Cove had the largest standing stock of understory in BMLR. VDSP had the highest algal cover of all three study areas. Surface canopy covered 65% to 79% of shallow and deep stations. Understory and foliose algae were more abundant at shallow than deep stations. PCMR was intermediate between BMLR and VDSP in macro-algae abundance, with the highest abundance at the shallow stations.

BMLR

Red abalone were low in abundance throughout BMLR. They were the most abundant at Horseshoe Cove (0.4 abalone m^{-2} SE = 0.1), followed by Cave (0.2 m^{-2} SE = 0.1) and Points (0.1 m^{-2} SE = 0.03) stations (Fig. 3). Almost all abalones were found at shallow stations, with only three encountered at deep (>8.4 m) stations. Red sea urchin densities were low in Horseshoe Cove (0.6 red urchin m^{-2} SE = 0.2) but higher at both the Cave (1.2 m^{-2} SE = 0.3) and Points (1.1 m^{-2} SE = 0.2). Purple sea urchins were found in very low abundance throughout this reserve. No significant correlation in counts was found between purple urchins and either red abalones or red urchins at any of the three BMLR sites. Red urchin and red abalone counts, however, were negatively correlated on transects at each of the three sites (Fig. 3). Plots of the distribution of counts by station showed marked segregation by species at almost all the stations, with few containing both species. Patterns of segregation were similar among the three locations in the reserve irrespective of relative density decrease for abalones or increase for sea urchins.

PCMR

While generally higher than BMLR, red abalone densities at PCMR declined in both shallow water (from 1.2 SE = 0.2 to 0.6 m^{-2} SE = 0.1) and in deep depths (1.2 SE = 0.2 to 0.1 m^{-2} SE = 0.1) during this 13-year interval (Fig. 4, Fig. 5). ANOVA comparison showed the difference in density to be significant by

TABLE 1.

Percent cover of encrusting organisms and macroalgae at Van Damme State Parke (VDSP), Point Cabrillo Marine Reserve (PCMR), and Bodega Marine Life Refuge (BMLR) for 1999.

	N	Encrust.	Turf	Foliose	Understory	Canopy
VDSP						
Shallow	21	62	39	41	53	65
Deep	11	44	18	31	30	79
PCMR						
Shallow	17	72	35	49	40	44
Deep	12	68	7	12	9	21
BMLR						
Horseshoe Cove	13	36	56	41	12	0
Cave	6	44	33	55	0	0
Points (NS)	12	41	24	33	3	0

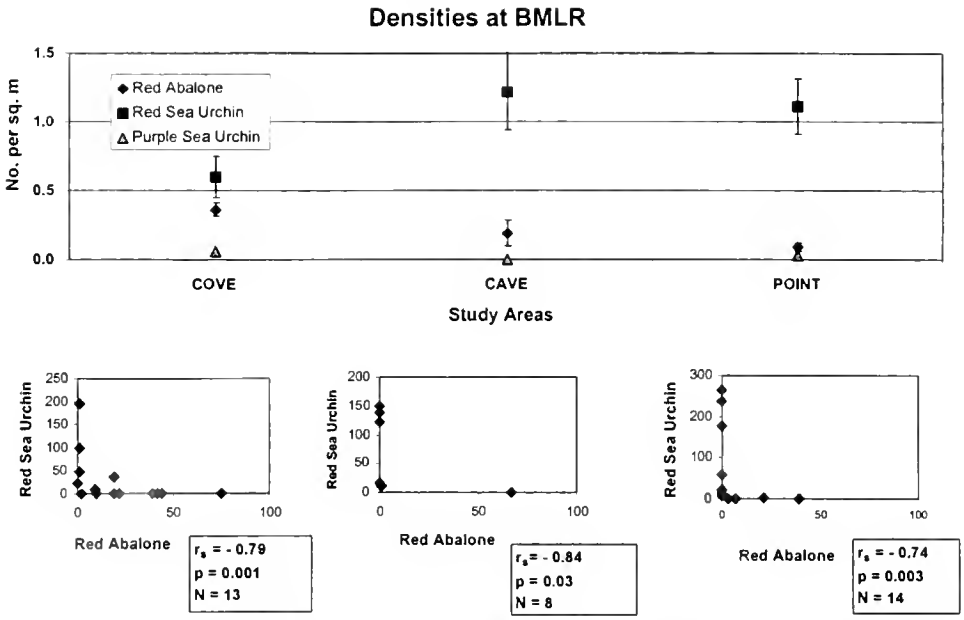

Figure 3. Red abalone, red sea urchin, and purple sea urchin densities (± se) by study area of BMLR with paired comparison of counts by species at each station with Spearman Rank correlation coefficients (r_s), statistical significance, and number of stations.

year, depth, and interaction (Table 2). Red sea urchin densities were significantly greater at depth and in 1999 (interaction was not significant). Size distributions reveal very little differences with large proportions of animals at both depths at or above legal sizes for both species (Fig. 6). Deep water red urchins showed evidence of recruitment in both years with bimodal distributions of YOY (<30mm) or small juveniles and large adults. Purple sea urchins, not surveyed in 1986, were at greatest abundance at shallow depths in 1999 (3.2 m^{-2}, SE = 1.0, Fig. 4 and Fig. 5).

Purple sea urchins at PCMR were not significantly spatially correlated to the other two species at the scale of our transects. Correlation comparisons between red abalones and red sea urchins were significantly negative in both 1986 and 1999, when shallow and deep stations were combined. Comparisons at shallow depths were significantly negative in 1986 and 1999 (Fig. 4). Graphical comparison of correlation revealed more overlap or concurrence of both species on the same transects than observed at BMLR. At deep stations in 1986 the correlation was negative but not significant (Fig. 5). PCMR was the only area where high densities of both red abalones and red urchins were found in our study. Under these high densities both species were significantly negatively correlated ($r_s = -0.52$ $P < 0.0001$, Table 3).

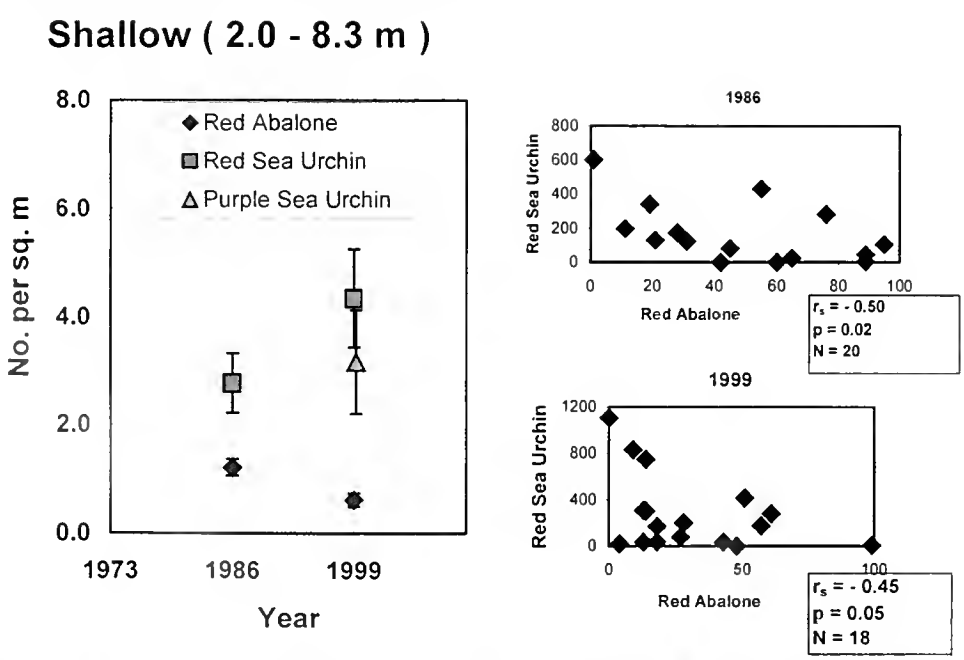

Figure 4. Red abalone, red sea urchin, and purple sea urchin densities (± se) at PCMR at shallow depth stations with paired comparison of counts by species at each station with Spearman Rank correlation coefficients (r_s), statistical significance, and number of stations.

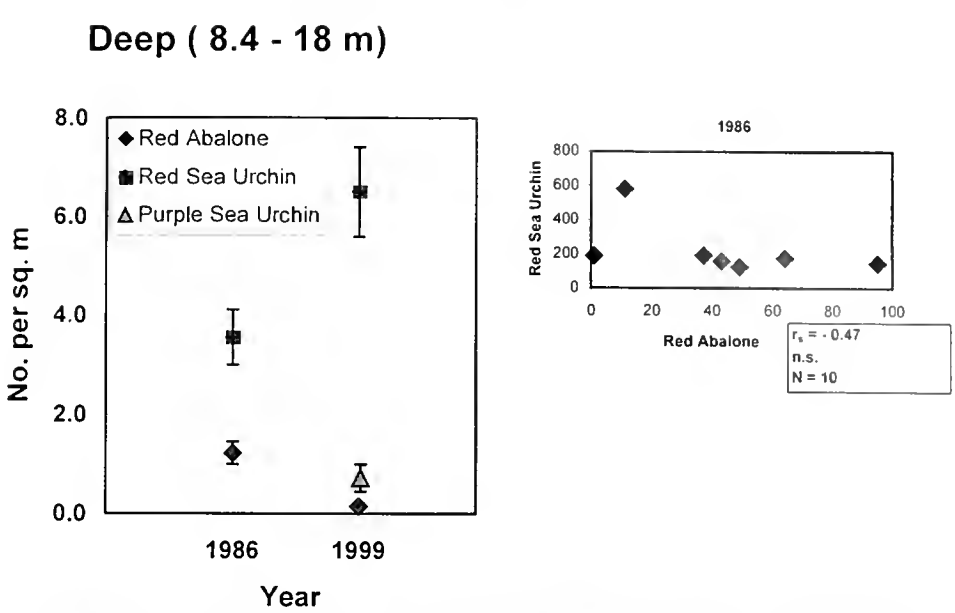

Figure 5. Red abalone, red sea urchin, and purple sea urchin densities (± se) at PCMR at deep depth stations with paired comparison of counts by species at each station with Spearman Rank correlation coefficients (r_s), statistical significance, and number of stations.

VDSP

In 1986 red abalone densities at VDSP were low in deep (0.1 m^{-2} SE = 0.1) and shallow depths (0.4 m^{-2} SE = 0.1), while red sea urchin densities were low at shallow (0.4 m^{-2} SE = 0.1) and high at deep stations (1.7 m^{-2} SE = 0.3) (Fig. 7, Fig. 8). Abalone densities had increased significantly to 0.8 (SE = 0.1) at shallow stations, and to 0.9 m^{-2} (SE = 0.2) at deep stations by 1992. Karpov et al. (1998) examined this difference using ANOVA and found the increase to be significant between years and depths (Table 2). During the same period, red urchin densities underwent significant decline by year and depth (P = 0.04 and 0.0001 respectively). By 1999, deepwater red urchin abundance had recovered to 2.4 m^{-2} (SE = 0.5), while red abalones had declined to 1986 levels (0.1 m^{-2} SE = 0.1). Repeating the ANOVA comparison revealed that the change in red urchin abundance was no longer significant by year, but still significant by depth. Purple sea urchins, first examined in 1989, underwent significant increases by depth and by year, increasing primarily at deep stations to 1.8 m^{-2} (SE = 0.7) in 1992 (Fig. 7, Fig. 8, Table 2).

VDSP data allowed comparison of species correlation at various combinations of density for each of the species (Table 3). Except at low densities of each, red abalones and red sea urchins were significantly inversely correlated ($P < 0.04$). Again, as in the other two study areas, no significant correlation was observed for purple sea urchins when counts were paired to either of the other two species.

Size distributions of both red abalones and red sea urchins at VDSP weighted for abundance were compared for evidence of recruitment by depth (Fig. 9). Red abalone size frequencies showed a clear pulse of juveniles at shallow depths in 1989. The mode in deep water appeared later, and grew into sizes larger than the sport size limit by 1992. In 1999, the deep water abalones remaining included few juveniles and resembled the 1986 and 1989 distribution. In shallow water, the distributions showed a large buildup of large adults over all previous years, with less evidence of recent juvenile recruitment than had been apparent in 1989 through 1992.

Size distributions of red sea urchins, first measured in 1990, showed a deep water peak at the minimum commercial legal size

TABLE 2.

Red abalone and red sea urchin two-way ANOVA probability values for log transformed density comparison by year, depth, and year*depth interaction at Van Damme State Park (VDSP) and Point Cabrillo Marine Reserve (PCMR). The first comparison at VDSP excludes 1999 data.

Comparison	Class Variable	Red Abalone	Red Sea Urchin	Purple Sea Urchin
VDSP (1986,1989–1992)	Year	0.004	0.04	0.03
(Shallow, Deep)	Depth	0.005	0.0001	0.0005
	Year*Depth	n.s.	n.s.	n.s.
VDSP (1986, 1982–1992, 1999)	Year	0.0008	n.s.	0.03
(Shallow, Deep)	Depth	<0.0001	<0.0001	0.0001
	Year*Depth	n.s.	n.s.	n.s.
PCMR (1986, 1999)	Year	<0.0001	0.008	
(Shallow, Deep)	Depth	0.029	0.001	
	Year*Depth	0.034	n.s.	

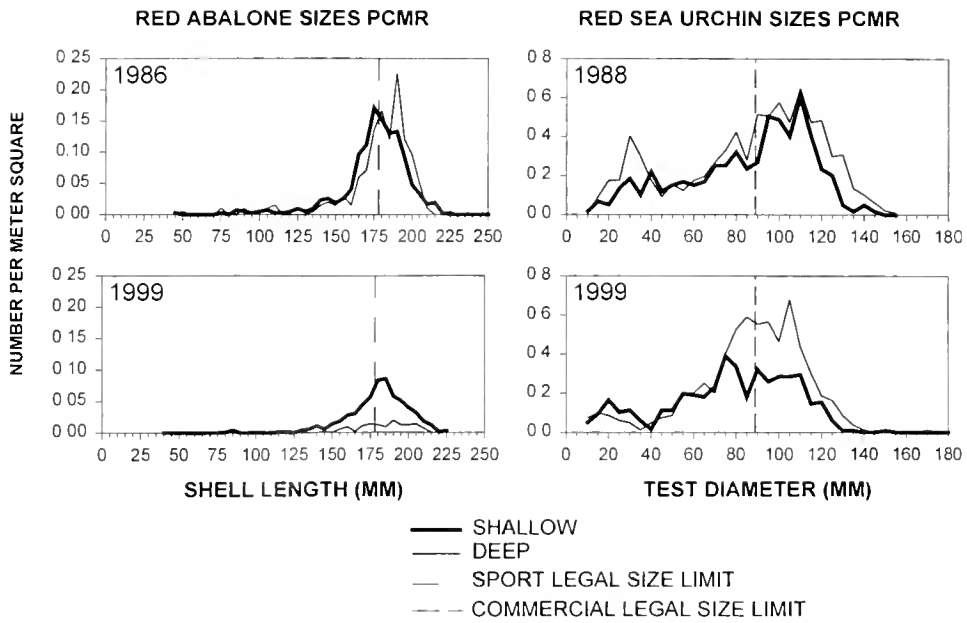

Figure 6. Size density of emergent red abalone and red sea urchin transects at PCMR at both shallow and deep depths. Years compared for red abalones are 1986 to 1999 and for red urchins 1988 to 1999. Vertical Lines represent minimum legal size for red abalone (178 mm) and red urchin (89 mm).

limit of 76 mm, first imposed in 1989 (Fig. 9). That mode declined through 1992 with little evidence of recruitment at either depth during that period. In 1999, a large cohort of red urchins, analogous to the earlier event for shallow water red abalones, had become established at deep depths with the peak, knife-edged, at the new 89 mm legal size limit, first imposed in June 1990.

Apparent Competitive Effects

Commercial landings of red sea urchins were first reported at the Van Damme area in 1985 (Fig. 10). Landings peaked at 800 mt in 1987, and declined along with CPUE (kg per diver day) through 1993. Size distributions of red urchins remained essentially flat from 1987 through 1990, becoming increasingly positively skewed by 1994 with few animals taken below the newly imposed size in 1990, and a decreasing number of animals larger than 120 mm taken (Fig. 11).

Surface kelp canopy at the VDSP area increased dramatically from 3 to 45 ha between 1982 and 1987. This coincided with increased red sea urchin removal (Fig. 10). *Nereocystis luetkeana* was the dominant kelp but the canopy also included *Macrocystis integrifolia*.

While the level of sport take was not monitored at VDSP, telephone surveys conducted from 1986 to 1989 estimated that there were 30,000 to 40,000 sportsmen in the northern red abalone fishery (CDFG unpublished). In 1998, a $12 stamp was instituted for the abalone sport fishery; 32,000 were sold in 1998, and 35,000 in 1999. VDSP is among the most highly utilized sport fishing sites in northern California. Creel surveys conducted at this site during spring and early summer since 1977 reveal continued high use with no decline in CPUE throughout our study period.

Red abalone size distributions from diver creels at VDSP can be segregated into pre- and post-onset of the sea urchin fishery. The first period, from 1977 through 1985, shows little change (Fig. 11). Size distributions during this period were highly skewed to the right, with the mode adjacent the sport legal size of 178 mm. Two of these years, 1983 and 1984, had the most positively skewed distributions with the lowest mean size of 188 mm. The second period (1986 to 1994) is characterized by dispersal into a flat distribution and an apparent modal progression from 182 mm in 1987 through 206 mm in 1992.

DISCUSSION

Spatial Interactions

There was a significant negative correlation between red abalones and red sea urchins at all sites and depths with the exception

TABLE 3.

Density matrix summarizing Spearman Rank Correlation test for red sea urchins compared to red abalones at low and high densities of each at Van Damme State Park (VDSP) and Point Cabrillo Marine Reserve (PCMR).

		Red Abalones	
Species	**Density**	**Low**	**High (>0.4 per sq. m)**
Red Sea Urchins	low	VDSP 1986 Shallow and 1989 Deep N = 29 n.s.	VDSP 1989–1992, 1999 Shallow and 1990–1992 Deep N = 101 r_s = −0.22 p = 0.003
	High (>1.0 per sq. m)	VDSP 1986 and 1999 Deep N = 18 r_s = −0.63 p = 0.005	PCMR 1986 and 1999 N = 60 r_s = −0.52 p < 0.0001

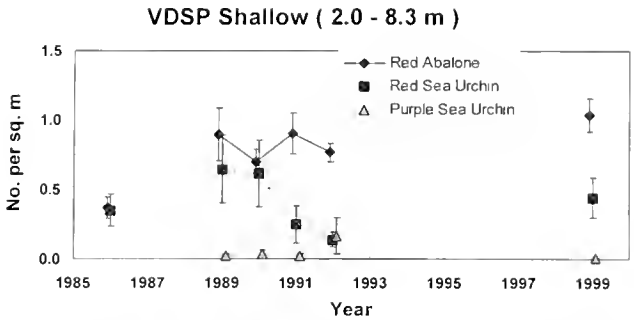

Figure 7. Red abalone, red sea urchin, and purple sea urchin densities (± se) at VDSP shallow depth stations in 1986, 1989 to 1992, and in 1999.

of deep-water stations at PCMR in 1999. This positive correlation is an artifact of data from only two stations; abalone were absent from 10 of 12 deep stations. The consistent negative correlation for all other comparisons suggests spatial interspecific exclusion between these two species. Deacon (1973) found a similar negative correlation between red abalones and red urchins in Sea Ranch, a northern California location between BMLR and VDSP. In 2000 we completed an additional 24 emergent transects, 13 deep and 11 shallow, at Salt Point State Park, located north of BMLR and south of VDSP (Bennett unpublished). Red urchins and red abalones were again negatively correlated in an area of high red abalone (0.84 m^{-2}) and low red urchin (0.90 m^{-2}) densities. Once more, red urchins were most abundant at deep and red abalones at shallow stations. Our finding of significant negative correlation at all but low densities of both red abalones and red urchins suggests that the observed spatial exclusion is density related. The increase in abalone abundance occurred during a period of increased surface

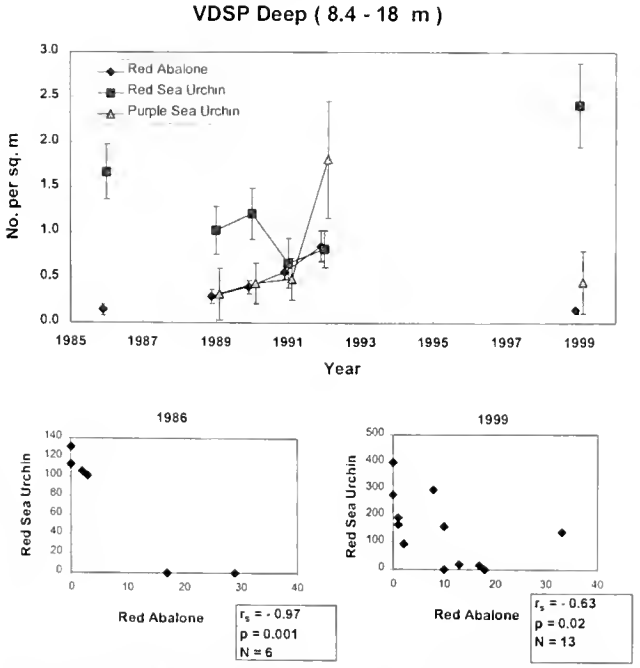

Figure 8. Red abalone, red sea urchin, and purple sea urchin densities (± se) at VDSP deep depth stations in 1986, 1989 to 1992, and 1999 with paired comparison of counts by species at each station with Spearman Rank correlation coefficients (r_s), statistical significance, and number of stations in 1986 and 1999.

canopy and therefore increased food abundance. Karpov et al. (1998) reported on the increase in abalone populations at VDSP at both depths from 1986 to 1992. Mechanisms responsible for this increase could include large-scale removal of potential competitors by the urchin fishery and positive effects on abalone growth due to increased kelp production.

The most profound lack of co-occurrence between red abalones and red sea urchins was at BMLR, the site with the least available macroalgae. This suggests competitive exclusion is greatest in habitat where food is limiting. It is likely that high silt loads from the Russian River (8 km to the north) and urchin grazing may limit kelp productivity in this area. Tegner et al. (1992) described areas adjacent to San Francisco Bay as marginal habitat, largely influenced by sediment loads from the bay. et al. (1997) reported comparable habitat to BMLR, with an absence of canopy and lack of understory algae below 13-m depth, off Fitzgerald Marine Reserve (another area near San Francisco Bay) in 1993. Canopy and understory kelps are the primary source of drift needed to support abalone as well as sea urchin populations (Deacon 1973, Tegner & Dayton 1991, Tegner et al. 1992).

The lack of correlation (positive or negative) between purple sea urchins and the other two species, at the scale of our transects, suggests that the spatial distribution of this species is not strongly correlated to the other two species. We note, however, that Schroeter (1978) found that the longer-spined red urchins exclude purple urchins from the most desirable habitat (within the m^2 scale) by spine fencing. The mechanism responsible for the increase in purple urchin abundance at both shallow and deep stations in VDSP may have been a release from competition for space as red sea urchin stocks declined due to fishing, or a strong coincident recruitment event. Off Sea Ranch, northern California, Deacon (1973) found a significant positive correlation between red urchins and purple urchins but no significant correlation between purple urchins and red abalones. Tegner et al (1989) observed significant increases in purple urchins at Johnson's Lee, Santa Rosa Island, an area that had also been subjected to intense fisheries for both red urchins and red abalones. Lafferty and Kushner (pers. comm.) reported periodic rapid increase and collapses of purple urchin populations off the northern Channel Islands in southern California from 1983 to 1998, with decreases due to starvation and disease following warming events. Our results and those of Tegner et al (1992) and Ebert and Russell (1988) are consistent with strong episodic recruitment for this species but do not clarify whether this follows release from competition for space.

Sea urchins are more adapted to periods of starvation than abalones, persisting for long periods on drift and microflora after macroalgae have disappeared from an area (Leighton 1968, Shepherd 1973, Tegner & Levin 1982). In spite of the overlap in their diet, red sea urchins have a more generalist feeding strategy than abalone (Leighton 1966). Abalones are essentially drift feeders, while sea urchins can act as drift feeders or can form feeding fronts that actively graze attached kelps when food is extremely limited (Harrold & Reed 1985). At both PCMR and VDSP, we found understory algae to be least abundant at deep depths, suggesting that food is more limiting at deeper depths. More drift algae at shallower depths have been observed at other sites in northern California (Rogers-Bennett et al. 1995).

The significant difference in red abalone and red sea urchin abundance by depth at VDSP and PCMR suggests differences in habitat preference between the two species. Greater numbers of red urchins were found at deeper stations and conversely greater num-

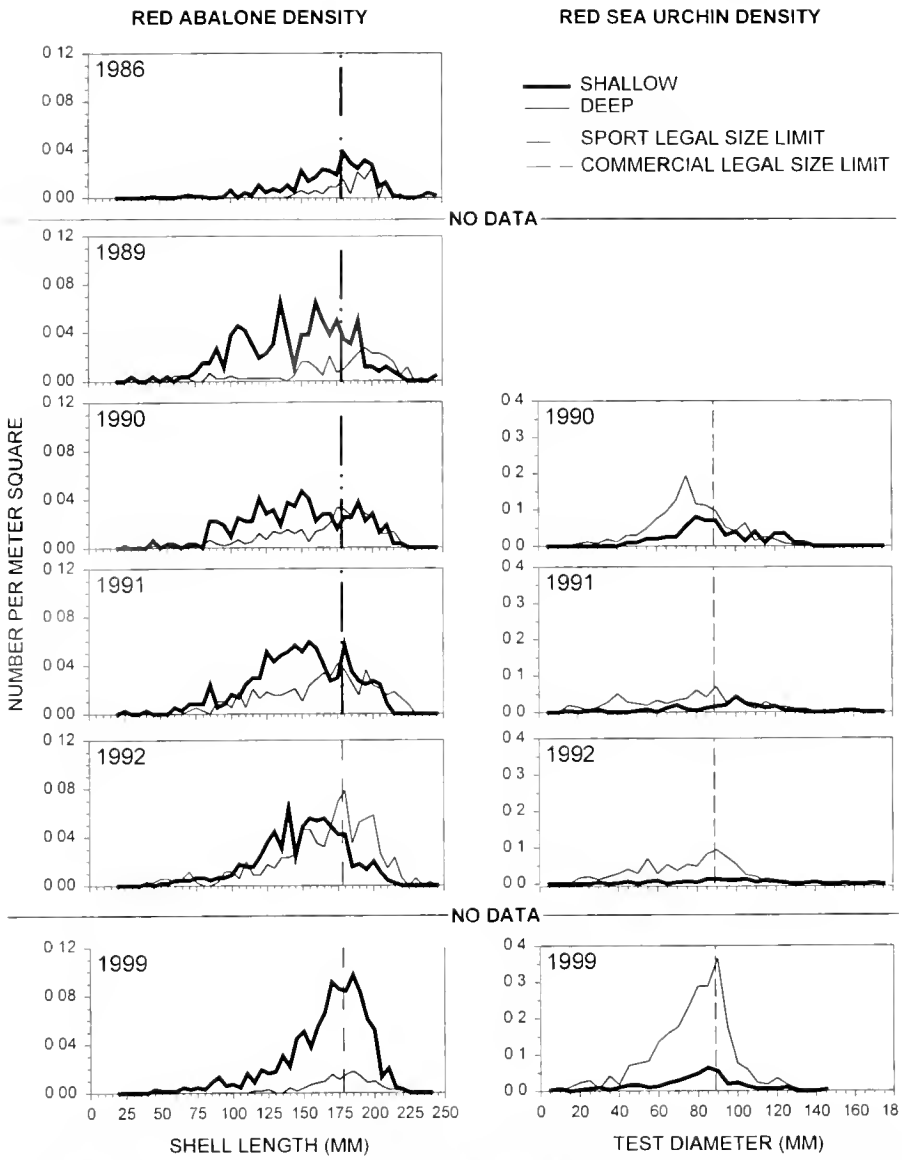

Figure 9. Red abalone and red sea urchin size density from emergent dive transects at VDSP at both shallow and deep depths. Years compared for red abalones are 1986, 1989-1992, and 1999; and for red urchins 1980-1992 and 1999. Vertical Lines represent minimum legal size for red abalone (178 mm) and red urchin (89 mm).

bers of red abalones at shallower depths. The absence of red abalones was most pronounced at deep depths off BMLR where food is most limiting, but was also apparent for abalones at VDSP, in spite of intense fishing pressure for abalone at shallow depths. The increased red abalone densities observed in deep stations at VDSP during the 1989 to 1992 period did not persist into 1999 and declined significantly following an increase in abundance of red urchins. A greater abundance of abalones at shallow depths in northern California may reflect their morphological adaptation to the high surge conditions found in shallow waters (Cox 1962, Deacon 1973). While both species prefer crevice habitats, especially in areas of high predation such as the sea otter range (Hines & Pearse 1982), urchins are more easily dislodged by wave action (Dayton 1973), and would be most vulnerable outside of crevices at shallow depths. In New Zealand, manipulative experiments reducing sea urchin densities enhanced the recruitment, survival, and growth of abalones (Andrew et al. 1998).

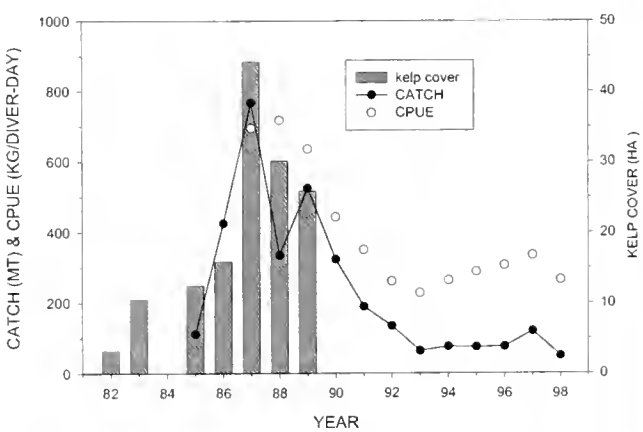

Figure 10. Commercial landings of red sea urchins, catch per unit effort (CPUE), and kelp canopy cover in the VDSP area.

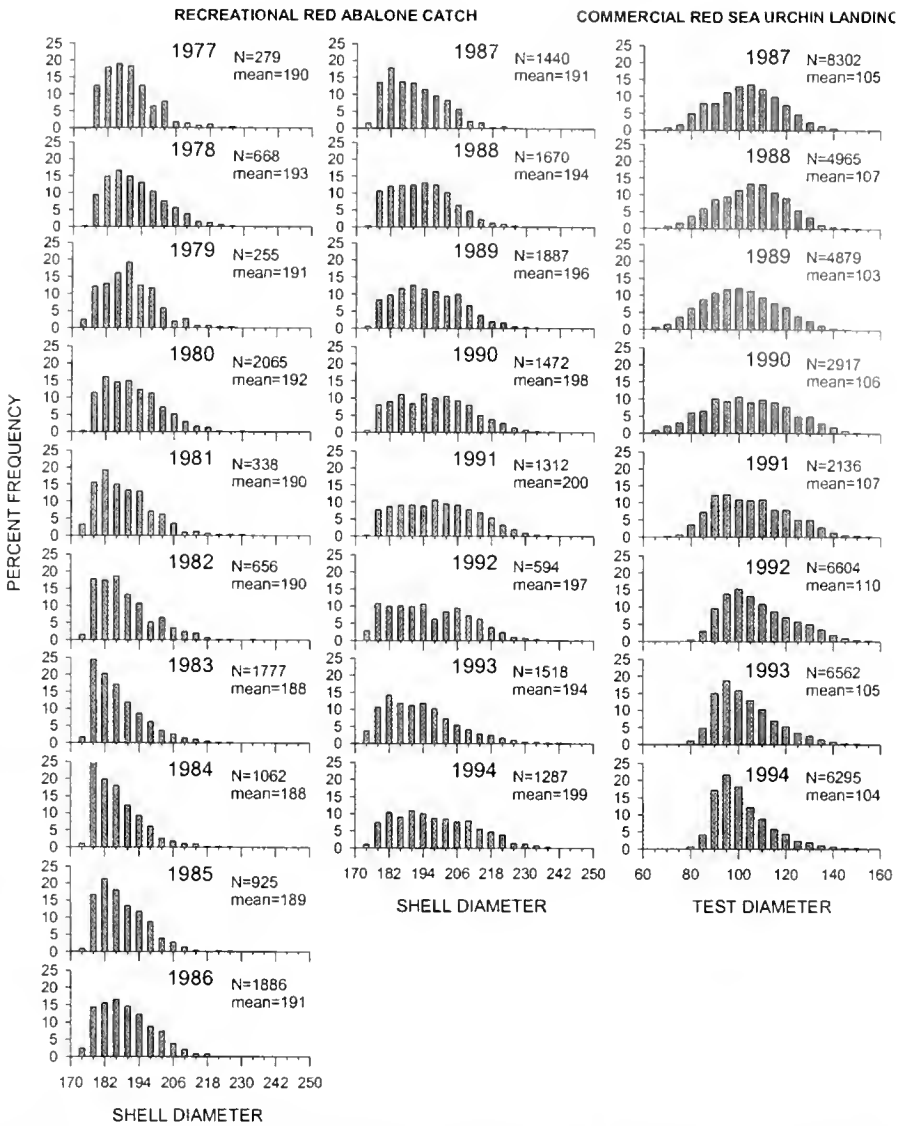

Figure 11. Size frequency distribution of commercial red sea urchins taken from the VDSP area from 1987 to 1994 with size of sport-diver-landed red abalones from 1977 through 1994. Legal minimum sizes are 178 mm for red abalones and 89 mm for red sea urchins.

Apparent Competitive Effects and Fishery Changes

Karpov et al. (1998) reported on the strong recovery in abalone populations in both depths at VDSP from 1986 to 1992. Both red sea urchin removal and enhanced abalone growth with increases in algal food are likely to have contributed to this increase in abalone abundance. Low kelp abundance observed at VDSP in 1982 and 1983 probably reflected the effect of grazing by an unfished population of red urchins. The rapid increase in kelp cover concurrent with high levels of urchin removal suggests kelp productivity and abundance increased along with removal of the sea urchins. Unfortunately, surface canopy aerial photographs were discontinued following 1989. Continued low levels of CPUE and increasingly skewed urchin size distributions in the fishery show that intense fishing continued throughout our study period.

Red abalone growth appears to be somewhat plastic and able to respond to increased food availability (Haaker et al. 1998). Modal progressions observed in size frequency distributions of sport-caught red abalones in the VDSP vicinity between 1987 and 1993 imply a growth rate of about 4 mm per year for abalone greater than about 180 mm shell length (SL) (Fig. 11). This growth rate is greater than observed in previous tag and recapture studies in northern California (Tegner et al. 1992) and southern California (Haaker et al. 1998). A DFG (unpublished) study at Van Damme from 1973 to 1977 based on 275 recaptured tagged red abalone showed an annual growth rate of <1 mm at sizes above 178 mm SL. While this is an area for more rigorous research, we propose a mechanism for this empirical observation of increased abalone growth at VDSP.

The importance of sea urchin grazing to the structure of kelp communities is well known (Nicholson 1970, Lawrence 1975, Harrold & Pearse 1987). Removal of an estimated 2,878 mt of red sea urchins from the VDSP area (approx. 3.5 km of coastline) from 1985 to 1993 coincided with an explosive increase in kelp canopy from 12.5 ha in 1985 to 44.2 ha in 1987 (Fig. 10). Concomitant with these events was a reduction in the larger size classes of red urchins. Red urchins ≥120 mm TD comprised 24.9% of the Mendocino County catch in 1992 (Mendocino County is used as a

proxy for the VDSP sea urchin catch, because of inadequate sampling at that location). By 1998, these sizes made up only 9.5% of the catch. This suggests a mechanism for the observed abalone growth rate increase, whereby removal of significant amounts of a primary macro-benthic grazer like sea urchins, as well as a continuing vigorous fishery for the abalones themselves, allowed release of the kelp canopy as well as subsurface kelps from grazing. This led to increased food availability for the remaining kelp grazers.

Red sea urchin size distributions by density at deep stations in 1999 showed peaks at 85–99 mm TD at PCMR and 80–94 mm TD at VDSP (Figs. 6 & 9). Based upon a combination of observational and experimental evidence, these peaks appear to be comprised primarily of a cohort that settled during the 1992–93 El Niño. Significant settlement events of red urchins were noted on artificial substrates monitored since 1990 at shallow subtidal stations at PCMR and Westport (about 30 km north of PCMR) in late spring and early summer 1992 and spring 1993 (Ebert et al. 1994). Subtidal surveys in the vicinity of PCMR in fall 1994 noted a strong cohort averaging 18 mm TD, 16 months following the spring 1993 artificial substrate settlement event (Kalvass & Hendrix 1997). Ebert (1997) developed a growth transition matrix for California red urchins with annual probabilities of transfer between 10 mm size groups. We applied these probabilities to the observed 1994 10–20 mm cohort and estimated that 63% would grow to the 80–100 mm size groups by the time we observed them in 1999 at both PCMR and VDSP. Growth of the observed cohort in 1994, from significant settlement events in 1992 and 1993, might account for much of the peak near the commercial red urchin minimum size limit of 89 mm TD observed in 1999 (Fig. 9).

Mechanisms

Our finding that red abalones are displaced from deep but not shallow depths at VDSP, combined with the negative correlation observed in abalones and red sea urchins at comparable densities in 1986, strongly suggests that competitive exclusion of red abalones has occurred. Disappearance of red abalone in deep water could have resulted from other mechanisms including illegal take (Daniels & Floren 1998), a major mortality event from disease (Lafferty & Kuris 1993), or movement to shallow depths during a period of successful red urchin recruitment.

Temporal changes in density at PCMR support the trend of exclusion of red abalones in deeper water but need to be interpreted cautiously. Densities where compared between two years bracketing a thirteen year interval and not a time series as sampled at VDSP. In addition, since only half of the same locations were resampled in 1999, spatial variations in habitat could have exaggerated (or underestimated) actual temporal differences in abundance (Thrush et al. 1994).

Red sea urchins in northern California appear to be much less mobile than red abalones. Ebert et al. (1999) tagged thousands of red urchins at 20 locations between southern California and southeast Alaska, for growth analysis. Recovery rates at these sites after approximately one year ranged from 13% to 76% for internally tagged urchins. These high recovery rates indicate that red urchins are relatively sessile. Conversely, red abalone tagging studies indicated abalone can move considerable distances. In a study at PCMR, Ault and DeMartini (1987) found that in an area of high abundance 11% of the tagged population moved over 90 m, with one animal moving 0.6 km. Similarly, high numbers of tagged red urchins (16–38%) were recovered after one year from sites in Salt Point in northern California. Twice as many urchins were recovered from a shallow site compared with a deep site, suggesting urchins in shallow water are more sedentary (Rogers-Bennett 1994). Deacon (1973) also found that the vast majority of red urchins moved far less than red abalone, during movement experiments at Sea Ranch. In the spring of 1998 starving red abalone were reported by divers south of Noyo Bay, 14 km north of VDSP (Haaker & Karpov, unpub. obs.). They observed a marked absence of understory kelps with evidence of abalone undergoing unusual movements over foliose algae in search of food. One possible explanation for the decline in deep-water abalones off VDSP could be recent movement to shallow depths in search of food.

Continued low numbers of red sea urchins at shallow depths in Van Damme are expected, given the high density of red abalone remaining at shallow stations in 1999. Urchins could only survive at shallow depth in crevice areas protected from extreme sea conditions (Deacon 1973), and it is unlikely that red urchins could displace red abalones from such habitat once occupied. While we found that the removal of red sea urchins at shallow depths in VDSP appeared to enhance the abundance of adult red abalones, juvenile abalones are known to shelter under the spine canopy of red sea urchins, complicating the relationship between red abalones and red sea urchins (Tegner & Dayton 1977, Rogers-Bennett & Pearse 2001).

CONCLUSIONS

Our results suggest that spatial competition between red abalones and red sea urchins is density related and appears to be most pronounced in habitats where macroalgae are scarce. In kelp beds these species co-occur on transects more frequently but are negatively correlated when either species is in high abundance. Intense red urchin fishing at VDSP coupled with a major recruitment event of red abalones appears to have enhanced abalone density in both shallow and deep depths through 1992. This release from inter and intraspecific competition for space and food resulted in a large surplus abalone population at refuge depths through the early 1990s. However, significantly lower densities of abalone are now apparent at depth. A major recruitment event for red sea urchins appears to have depressed abalone populations at depth. We cannot rule out the possibility of movement, mortality, or poaching of deep water red abalones as causes for the decline. In the absence of fishing for both species, densities of red abalones at an adjacent PCMR reserve site are now below those found at the intensely fished area, while sea urchins have increased to greater numbers than fished abundance. With its high urchin abundance, PCMR kelp populations are also lower than at nearby VDSP. Together this suggests that fishing of both red urchins and red abalones at VDSP enhanced abalone densities. We therefore suggest that an ecosystem approach that takes into account multispecies interactions, should be an important consideration in managing these fisheries in northern California.

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CLIMATE VARIABILITY, KELP FORESTS, AND THE SOUTHERN CALIFORNIA RED ABALONE FISHERY

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ABSTRACT Declines in landings of Southern California abalone fisheries and the eventual collapse of many stocks over the last two decades coincided with a period of greatly increased environmental variability. This included massive storms, an increase in the frequency of warm-water El Niño events after 1976, and an interdecadal-scale increase in sea surface temperatures. Kelp populations may be decimated by severe storms or warm water. Because of the strong inverse relationship between nitrate availability and water temperature, temperature is a good indicator of nitrate availability or stress SINCE kelp growth ceases in warm nutrient-depleted water, tissue decays, and standing stocks may be greatly reduced. Abalones are affected by the availability of the drift kelp on which they feed. Anomalously warm temperatures may affect reproduction, and altered current patterns may affect larval dispersal. Because water temperature varies with location in Southern California and each of the five exploited abalone species has its own thermal preferences, we chose to evaluate the role of environmental variability on populations of red abalone (*Haliotis rufescens*) on three northern Channel Islands spanning a temperature gradient. We compared water temperature regimes and anomalies, monthly aerial surveys of canopies of giant kelp (*Macrocystis pyrifera*), and field evidence of poor abalone growth and reproduction during El Niño events. The severity of El Niño disturbances and long-term changes in kelp standing stocks both correlated with the temperature gradient. Declines of red abalone total landings and area-specific landings on the warmer Santa Cruz and Santa Rosa Island began a decade after the large 1957–1959 El Niño. The subsequent collapse of many populations appears related to warm anomalies after the 1976–1977 regime shift, kelp declines, and poor reproduction coupled with fishing-induced declines in adult abalone density. Red abalone populations have persisted on cooler San Miguel Island where thermal anomalies had less effect and kelp canopy biomass has been more stable. Southern California abalones evolved in this disturbance regime, but the combination of extended periods of increased environmental variability with intense fishing pressure may have led to the loss of local populations, especially in warmer areas.

KEY WORDS: El Niños, *Macrocystis*, storms, ocean climate, reproduction, stock collapse

INTRODUCTION

Declines in the landings of Southern California abalone fisheries and the eventual collapse of many abalone stocks over the last two decades (Karpov et al. 2000) coincided with a period of changing ocean climate (McGowan et al. 1998). Since 1976 to 1977, there has been an increase in the frequency, duration, and intensity of warm-water El Niño events and a corresponding decrease in cold-water La Niña events (Fig. 1). Frequently described as a regime shift, this has been accompanied by a significant interdecadal-scale increase in sea surface temperature (Roemmich & McGowan 1995), and a shifting in the mean location of sea surface isotherms to the north along the West Coast of North America (Fig. 2). Furthermore, large waves in Central and Southern California are strongly associated with El Niño events, and there has been a marked increase in the number of broad area wave events exhibiting very large waves (Seymour 1996).

El Niños and large wave events are important agents of disturbance to kelp forest communities, with severities that vary with latitude and exposure (Tegner & Dayton 1987, Seymour et al. 1989). El Niño storms, such as those that produced the extraordinary number of large wave events in winter 1983, may decimate exposed kelp populations along the entire coast of California. Storms also kill abalones directly (Cox 1962). The effects of the warm, nutrient-depleted waters, however, are stronger in the southern portion of the range of giant kelp, *Macrocystis pyrifera*, the major source of productivity and structure in this community (Tegner & Dayton 1987). Sea surface temperature is the best predictor of harvestable *Macrocystis* biomass (Tegner et al. 1996). Because of the strong inverse relationship between nitrate availability and water temperature, temperature is a good indicator of nitrate availability or stress, as kelp growth ceases in warm nutrient-depleted water, tissue decays, and standing stocks may be greatly reduced. Community effects of the 1982 to 1984 El Niño were apparent long after the anomalous oceanographic conditions, as manifested by increased abundance of understory kelps relative to that of *Macrocystis* (Tegner et al. 1997). The long-term increase in temperatures since 1977 is associated with a 2/3 decrease in the median size of giant kelp plants (measured as a decline in number of stipes) and thus a decrease in drift kelp availability (Tegner et al. 1996). Furthermore, because strong El Niño events are adding to the secular increase in ocean temperatures, the 1997 to 1998 event was warmer than the very strong 1982 to 1984 event.

While the effects on kelps are relatively well understood, the impacts of these changes in ocean climate on consumers are poorly known. Temperature affects animals directly, and temperature and storms both affect the food supply of grazers. The west coast of North America supports eight species of abalones, and their geographic and depth distributions are largely determined by temperature (Leighton 1974). Thus, effects may be apparent in distribution as well as physiology. *Macrocystis* is the dominant source of drift kelp in Southern California (Tegner & Dayton 1991), and the major food for abalones (Tutschulte 1976). With up to 60% of the biomass of a healthy *Macrocystis* forest in its canopy (North 1968), the loss of that canopy and varying degrees of plant mortality lead to a corresponding decrease in drift availability. Understory kelps produce far less drift per unit area than *Macrocystis*. Drift availability is very low after major storm disturbance, juvenile giant kelp populations produce little drift, and thus drift production may remain low for months to years after an El Niño event (Tegner & Dayton 1991). Furthermore, warm, nutrient-depleted

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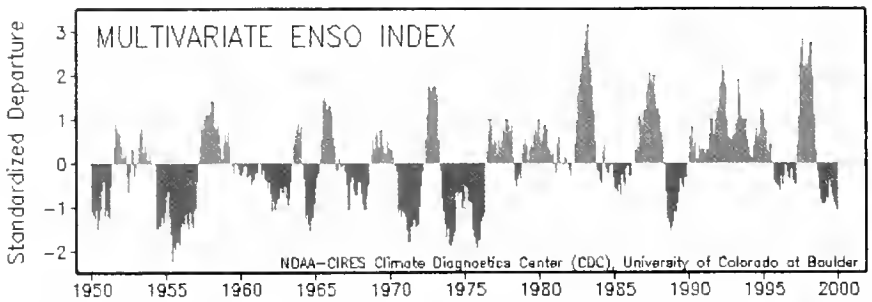

Figure 1. Multivariate El Niño-Southern Oscillation Index (courtesy of National Oceanographic and Atmospheric Administration's Climate Diagnostic Center). Downloaded in January 2000 from http://www.cdc.noaa.gov/ENSO.mei_index.html). El Niño events are positive, La Niña events are negative. This is a tropical index; events strong enough to affect the California Current region exceed some threshold about the mean.

conditions also lead to a decrease in the nitrogen content of kelps (Tegner & Dayton 1987), resulting in both the quantity and quality of abalone food being altered.

Previous observations indicate that abalones are affected by environmental variability. Cox (1962) noted that "during 1957, 1958, and 1959, the main food supply of abalones off California practically disappeared when an unseasonal influx of warm water destroyed all of the kelp beds. During this starvation period, the gonads of the abalones did not increase in size during their regular spawning season and many probably were unable to spawn." He also observed that growth was minimal or nonexistent, and that body tissues appeared to shrink. The flesh of abalones that did enter the fishery was shrunken and watery. Haaker et al. (1998) later quantified strong interannual variability in growth rates, including a dramatic reduction in the growth rate of *Haliotis rufescens* (red abalone) during the 1982 to 1984 El Niño event. Vega et al. (1997) observed an inverse correlation between sea surface temperature anomalies and total catch of abalones from Baja California which they related to impacts on algal food abundance. On the other hand, Shepherd et al. (1998) found total abalone catch at La Natividad, Mexico, to be positively correlated with mean sea surface temperature anomalies with a lag of eight years, but also reported evidence for recruitment failure during severe El Niños.

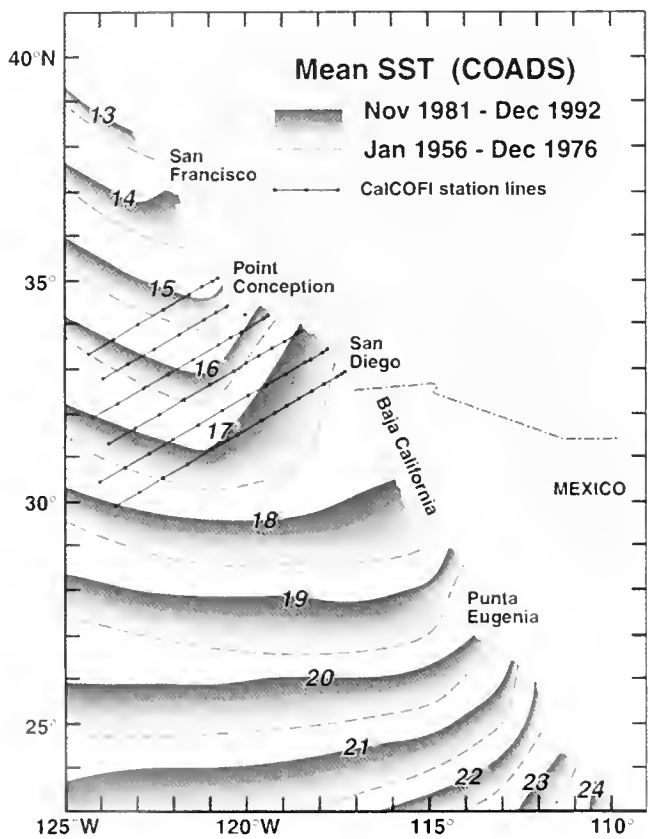

Figure 2. Changing isotherm locations along Baja California (Mexico) and California (USA). The Southern California Bight extends from Point Conception to just below the Mexican Border. The present California Cooperative Oceanic Fisheries Investigations (CalCOFI) grid is illustrated by station lines. The range of *Macrocystis pyrifera* is from just south of San Francisco to the region just south of Punta Eugenia. COADS (Comprehensive Ocean Atmosphere Data Set, http://www.cdc.noaa.gov/coads/) data were used to determine the location of the annual mean sea surface temperature (SST) isotherms before and after the mid 1970s regime shift. The dashed line represents the position 1956 to 1976 and the solid line represents 1981 to 1992.

Field studies on the Palos Verdes Peninsula (33°46′ N, 118°25′ W) near Los Angeles during and after the strong 1982 to 1984 El Niño event suggest that anomalies in both temperature and food availability affect abalone reproduction and recruitment. We followed gonadal development of green (*H. fulgens*) abalones from February 1982 through September 1983. Normally these animals spawn in both the spring and the fall. El Niño storms in January 1983 removed all *Macrocystis*; the abalones had little if any food until kelp recruitment in May. Gonadal development in spring 1983 began much later than in 1982, was very low in comparison with the previous year, and it is unlikely that there was significant spawning (Tegner & Dayton, 1987, Fig. 9). The green abalone is a warm-water species near the northern end of its range at Palos Verdes (Cox 1962) and winter-spring 1983 was before the major temperature anomalies associated with this ENSO; thus the observed depression in reproduction may have been primarily caused by lack of food. Size-frequency distributions of cool-temperate red and warm-temperate pink (*H. corrugata*) abalones collected in 1986 at Palos Verdes suggested that El Niño conditions affected these congenors quite differently. There was a near absence of two and three year old red abalones, animals that would have been spawned in 1983 and 1984; virtually all the pink abalones were in these size categories (Tegner & Dayton, 1987, Fig. 10). These data suggest that temperature anomalies may have favored pink abalones over the colder water reds during the ENSO event. We cannot exclude large-scale alterations in current patterns during El Niño events (Tegner & Dayton 1987) also affecting abalone recruitment, but this seems unlikely given the short larval period (McShane 1992). On smaller scales, the devastation of kelp forests and con-

comitant reductions of their effects on flow could increase local dispersal of larvae (Tegner & Dayton 1987).

While it is likely that all abalone species are affected by variability in ocean climate, the effects will vary with species and location. Because Southern California is a mixing ground between cool temperate waters of the California Current coming from the north and warm-temperate flow from the south (Fig. 3), comparisons of how populations in thermally distinct habitats respond to interannual variability may offer insights into the relative effects of different factors. Here we compare red abalone populations across the thermal gradient from colder San Miguel Island to Santa Rosa Island to warmer Santa Cruz Island. We use long-term records of temperature, kelp canopy biomass, field assessments of abalone populations, and fisheries statistics to explore hypotheses relating environmental variability to the decline of the red abalone fishery and the collapse of many local stocks.

MATERIALS AND METHODS

Historically the red abalone is the most important species in the California abalone fishery by virtue of its large size, wide distribution, and long harvest history. Its range extends over 16° of latitude from Coos Bay, Oregon to Bahia Tortugas, Mexico (Cox 1962). From Southern California south, however, it is limited to the northwestern Channel Islands which are heavily influenced by the cold waters of the California Current (Fig. 3), and to mainland locations where strong upwelling moderates temperatures (Tegner et al. 1992). Leighton (1974) reports that he found fecund, spawnable red abalones every month of the year (1969–1971) at Estero Bay in Central California, whereas in Southern California he obtained laboratory spawnings in January, February, April, September, November, and December. Before the fishery was closed in 1997, size limits were 178 mm and 197 mm for the recreational and commercial harvests, respectively.

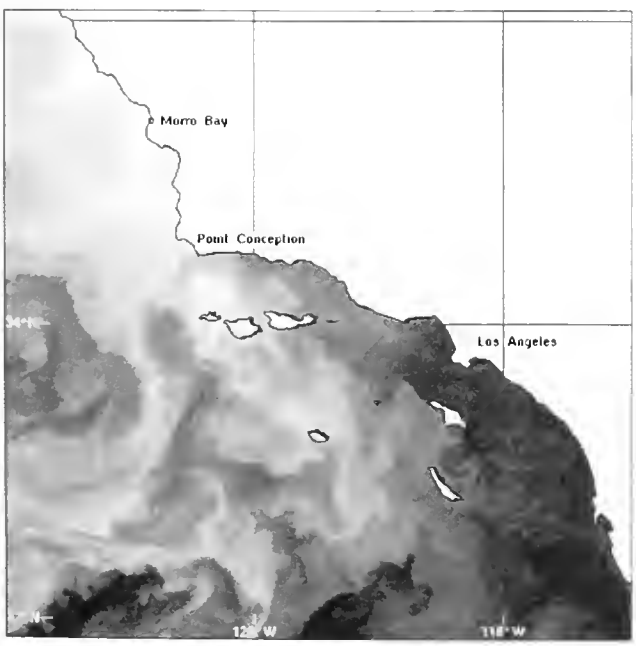

Figure 3. This satellite image illustrates thermal variability along the Central and Southern California coastline on November 21, 1996. The coldest temperatures are white and the warmest are black. Note the cold waters of the California Current flowing south past Point Conception and the warm waters of the Southern California Eddy flowing north along the mainland; mixing of these two water masses creates temporal and spatial variability within the Southern California Bight. The three islands immediately below Point Conception are, from west to east, San Miguel, Santa Rosa, and Santa Cruz Island. NOAA AVHRR (Advanced Very High Resolution Radiometer) sea surface temperature data provided by the NOAA Coastwatch Program for and displayed in Windows Image Manager.

Channel Island sea surface temperature data (courtesy of Jack Engle, Tatman Foundation, University of California, Santa Barbara) were generated from regional satellite-derived isotherm maps of the California coast posted several times a week by the National Weather Service (http://www.nws.mbay.net/sst1.gif). Data are interpolated to 0.5 C, and averaged by month for each island. Mean monthly temperatures calculated for the period 1982–1998 were used to determine temperature anomalies through time.

Biologists from International Specialty Products (formerly Kelco) have been making approximately monthly aerial estimates of *Macrocystis* biomass available for harvesting (California law allows removal of the upper 1.2 m of the canopy) for more than three decades. As these estimates are made by a very small number of observers and continually refined against the actual harvest tonnage, we believe that these estimates are quite accurate. There were no estimates made during 1992 and 1993. To protect proprietary harvest information and to eliminate the effect of the large differences in size among the islands, data are presented as a proportion of the maximum observed in the time series for each island. Linear models for all three islands were fitted with the percent of maximum annual relative canopy biomass as the response variable and year as the predictor variable. The error distributions of the relative annual canopy biomass data were determined to be normal according to visual analysis of residual-fit spread plots and normal quantile plots of residuals. These assured that all of the three fitted models adequately explain the variation in the data (Cook & Weisberg 1982, McCullagh & Nelder 1989).

Sex ratio was assessed visually (Ebert & Houk 1984) at San Miguel Island in May 1997. Red abalones of varying sizes were collected from several locations on San Miguel Island with a target goal of 30 per sample. Animals were brought to the surface and gonad color was scored by at least two observers: dark colored (green to black) for females and light colored (cream to light brown) for males.

Size-frequency distributions of red abalones from a single location on Santa Rosa Island and from four locations on San Miguel Island were collected in June 1999. The data were summed for the San Miguel Island sites.

RESULTS

Temperature Records

Mean monthly temperatures for the three islands for the period 1982 to 1998 are plotted in Figure 4. It is apparent that San Miguel Island is consistently colder than Santa Rosa Island, which is consistently colder than Santa Cruz Island. The differences are minimal in the winter and maximal in late summer. Monthly averages were compared using Friedman's two-way ANOVA. The results showed significant differences between sites ($P < 0.001$) and an a posteriori test (Nemenyi 1963) indicated that each island's temperature regime was significantly different from the others'. Note that this data set begins after the 1977 regime shift.

Time series of ambient temperature in comparison with the mean monthly values and the anomalies for 1982 to 1998 are

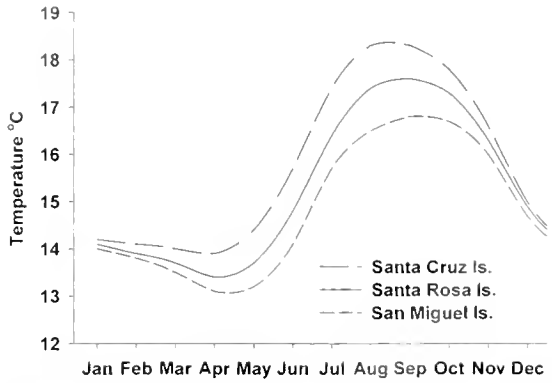

Figure 4. Mean monthly temperatures, 1982 to 1998, for San Miguel, Santa Rosa, and Santa Cruz Island.

plotted in Figure 5. Note the 1982 to 1984, 1992 to 1993, and 1997 to 1998 El Niño warm anomalies. La Niña events took place in 1988 to 1989 and 1999 (Fig. 1). The timing and magnitudes of the anomalies were remarkably similar among the three islands, but the summer values were higher at Santa Cruz Island and the winter values lower at San Miguel Island because of the difference in average temperatures.

Macrocystis Canopy Biomass

Time series of aerial estimates of giant kelp canopy biomass (Fig. 6) exhibit the strong interannual variability characteristic of *Macrocystis* populations (e.g. Tegner et al. 1996). Many of the peaks and depressions are common to all three islands, for example the regrowth in 1984 and 1985 after the winter 1983 storms and the 1983 to 1984 El Niño warm anomaly disturbances. Because some of the within-year decreases in canopy biomass are due to natural disturbance and some to harvesting, the annual maximum biomass estimates for each island are compared in Fig. 7. Some of the patterns are strikingly similar in timing and magnitude, including the decline in canopy at all three islands from 1978 to the low 20% of maximum biomass from 1986 through 1999. San Miguel Island, the coldest of the three, had a higher relative canopy biomass than intermediate temperature Santa Rosa Island from 1995 through the end of 1999.

Regressions of maximum annual relative canopy biomass against time were used to determine whether (a) kelp populations on the different islands had declined, (b) whether the regression coefficients changed at the time of the 1976 to 1977 regime shift, and (c) whether the islands were different from each other. (a) Analysis of variance indicated that two of the three slopes were significantly different from zero. Relative canopy biomass declined significantly at Santa Rosa Island (slope −0.011, $r^2 = 0.19$, $P = 0.04$), and at Santa Cruz Island (slope −0.014, $r^2 = 0.28$, $P = 0.001$) from 1968 to 1999. (b) T tests comparing regression coefficients before and after the 1976 to 1977 regime shift were not significant for any island. (c) Analysis of covariance, however, found significant differences in the elevations of the regressions ($P = 0.02$). Multiple comparisons (Tukey test) of the elevations of the regressions indicated that San Miguel Island canopy biomass was significantly different from that at Santa Cruz Island, but Santa Rosa Island canopy biomass was not significantly different from either San Miguel or Santa Cruz Island canopy biomass. Thus, kelp canopy biomass has persisted better at San Miguel Island relative to Santa Cruz Island.

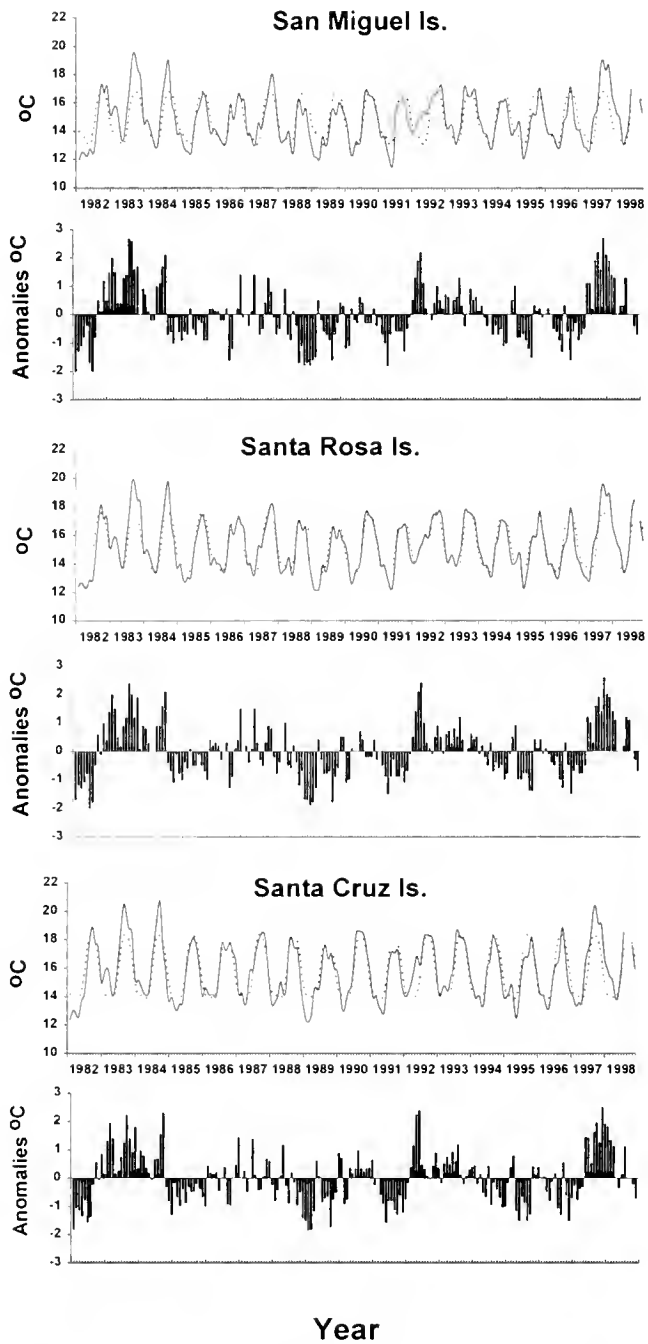

Figure 5. Time series of monthly average temperatures (solid lines) in comparison with mean monthly temperatures (dotted lines) for the period 1982 to 1998 (upper panels). Monthly anomalies (bars) from mean monthly temperatures, 1982 to 1998 (lower panels). See text for details. San Miguel, Santa Rosa, and Santa Cruz Island.

Abalone Field Data

In May 1997, red abalone sex ratios (assessed visually) on San Miguel Island were heavily skewed toward females. The sex ratio was 5.8:1 (female:male) on the west side of the island ($n = 34$), 5.7:1 on the south side of the island ($n = 60$), and 5.6:1 on the north side ($n = 33$). At this time near the onset of the 1997 to 1998 El Niño, the surface temperature on San Miguel Island was about 1°C above normal (Fig. 5) and kelp canopy biomass was only 10%

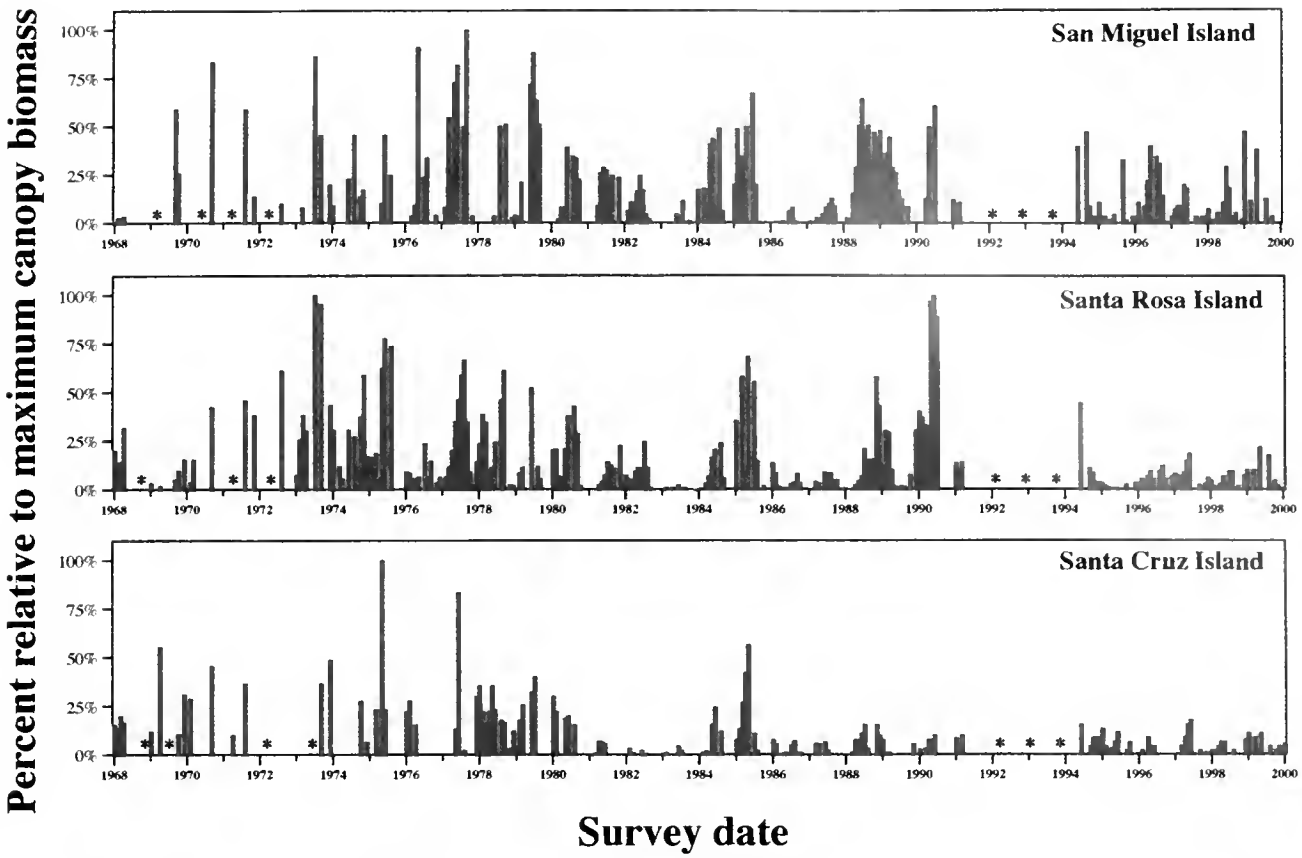

Figure 6. Monthly aerial estimates of *Macrocystis pyrifera* canopy biomass from San Miguel, Santa Rosa, and Santa Cruz Island An asterisk (*) indicates no survey.

of maximum (Fig. 6). The sexes of 15 animals could not be determined. Observers noted that sex determination was more difficult than in February 1997, when temperatures were slightly below the long-term mean.

The red abalone size-frequency distribution on San Miguel Island in June 1999 was strongly bimodal (Fig. 8). The near absence of sizes representing two and three year old individuals suggests poor reproduction and recruitment in 1997 and 1998. The strong mode at 20 mm suggests that reproduction quickly followed the onset of the La Niña and cooler temperatures in November 1998; the kelp canopy recovered to 47% of maximum by January 1999 (Fig. 6). Limited size-frequency data collected at San Miguel Island in 1993 revealed very few recruits; a collection from Castle Rock on western San Miguel Island in 1994 after the 1992 to 1993 El Niño found a burst of small animals (Haaker unpub. data). The small 1999 sample from Santa Rosa Island, where red abalone densities are very low (Fig. 8), suggests that reproduction and recruitment have not been successful on this island for the last four years.

DISCUSSION

Snapshots of sea surface temperature (Fig. 3), mean monthly temperatures (Fig. 4), and biogeographic differences in species composition (e. g., Murray et al. 1980) all indicate that there is a significant thermal gradient across the northern Channel Islands from coldest at San Miguel Island in the west to warmest at Santa Cruz Island in the east. Temperatures are highly variable in time, but the data in Figure 5 suggest that approximately constant average temperature differentials among the three islands are maintained through El Niño and La Niña events, as well as interdecadal scale changes. Temperature is directly important to abalone physiological processes and larval survival (Leighton 1974), to harvestable *Macrocystis* biomass (Tegner et al. 1996), and to the quantity and quality of drift *Macrocystis* (Tegner & Dayton 1987), the major food source of abalones (Tutshulte 1976).

Because of the strong inverse relationship between temperature and nitrogen availability, *Macrocystis* populations are very sensitive to extended periods of warm water. Warm water *per se* does not harm giant kelp which, if provided with abundant nutrients, grows well even at 25°C (reviewed by North & Zimmerman 1984). There are negligible amounts of nitrate above 15°C (Jackson 1977, Gerard 1982, Zimmerman & Kremer 1984) but giant kelp thrives at 14°C, so very small differences in temperature can have important community effects. *Macrocystis* can build internal N reserves when external concentrations are high, and then use these reserves to maintain relatively rapid growth for up to a month in the absence of significant external nutrients (Gerard 1982, Zimmerman & Kremer 1986). When kelp tissue N drops below 1% (dry weight), internal reserves are depleted, and N starvation will lead to rapid deterioration. During most summers when surface temperatures exceed 16°C, upwelling and internal wave-induced thermocline motions provide nutrient input to maintain giant kelp populations (Zimmerman & Kremer 1984). During strong El Niño events, the thermocline is often depressed to levels too deep for

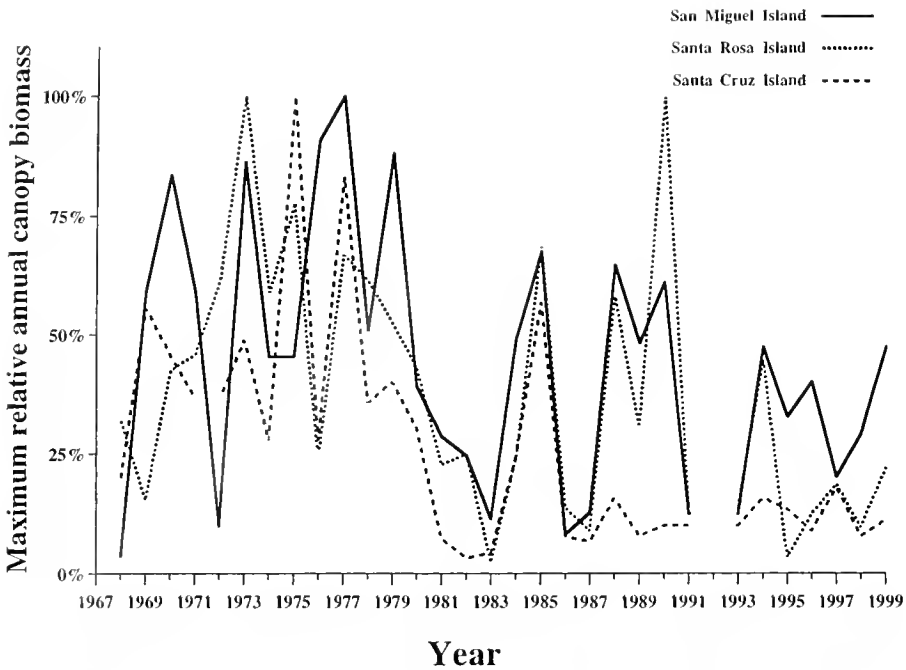

Figure 7. Maximum annual estimates of *Macrocystis* canopy biomass from San Miguel, Santa Rosa, and Santa Cruz Island.

these processes to be effective (Zimmerman & Robertson 1985). *Macrocystis* canopies were completely lost on the southern-most Santa Catalina and San Clemente Islands and along the southern-most counties, Orange and San Diego, in California during the 1982 to 1984 El Niño (Tegner & Dayton 1987). Further to the north, given temperature decrease with latitude, the warm anomalies added to lower temperature bases and there was some degree of giant kelp canopy formation. Conversely, *Macrocystis* thrives during the cold water and enhanced upwelling of La Niña events (Tegner et al. 1997).

Santa Cruz, Santa Rosa, and San Miguel Island experienced temperature anomalies during recent El Niño events that were remarkably similar in timing and magnitude; the difference is that the anomalies added to a different temperature base at each island. Thus, the anomalies were more likely to make the environment unsuitable for *Macrocystis* at Santa Cruz Island, and that may explain low standing canopy biomass estimates at that island from the mid 1980s through 1999 (Figs. 6, 7). There were significant declines in relative canopy biomass at Santa Cruz and Santa Rosa Island, but not at cooler San Miguel Island. We stress, however, that aerial surveys cannot detect sea urchin barrens, another potential explanation for poor performance of giant kelp. Because up to 60% of the biomass of a healthy *Macrocystis* population is in the canopy (North 1968), we can safely infer that poor canopy condition is reflected in low drift availability (Tegner & Dayton 1991) for grazers.

Several lines of evidence suggest that red abalone reproduction and recruitment are depressed by warm temperatures and lack of food. Cox (1962) reported the lack of gonad development and the low probability of spawning during 1957 to 1959, a warm anomaly that only later would be recognized as a strong El Niño event. There was a near absence of animals of the size classes that would have been spawned in 1983 and 1984 in a 1986 size-frequency distribution from the Palos Verdes Peninsula (Tegner & Dayton 1987). Visually assessed sex ratios from San Miguel Island were highly skewed toward females in 1997, near the onset of the 1997 to 1998 El Niño. A 1:1 sex ratio is expected in abalones, but when gonads are poorly developed, it is impossible to distinguish the grayish-brown digestive gland from an enveloping dark-green ovary (Ebert & Houk 1984). Later histological examination of 97 of these animals revealed a 1:1 sex ratio (C. S. Friedman unpub. data), indicating that many adult red abalones stressed by environmental conditions at San Miguel Island had undeveloped gonads. Finally, size-frequency data from San Miguel Island in 1999 (Fig. 8) suggests little to no recruitment during the 1997 to 1998 El Niño. Warm temperature anomalies of the magnitude observed in 1983 to 1984 and 1997 to 1998 were also observed in 1992, and that anomaly persisted into early 1994 (Fig. 5). Furthermore, McGowan et al. (1998) illustrate regional sea surface temperature anomalies of about 2°C in 1977 and 1978, and about 1°C in 1980, a period of the decline in *Macrocystis* canopy biomass at all three islands (Fig. 7). Thus, there have been a large number of warm events since 1977 that may have affected red abalone reproduction and recruitment.

Haaker et al. (1998) assessed the annual growth of tagged red abalones on Santa Rosa Island from 1978 to 1982, and revisited the site in 1984, a two year hiatus that included the strong 1982 to 1984 El Niño event. Growth parameters changed significantly throughout the study period, with L_∞ generally declining during each successive period. Warm anomalies in 1977, 1978, and 1980 (McGowan et al. 1998) followed by the 1983 El Niño winter storms (Tegner & Dayton 1987) were associated with a substantial decline in *Macrocystis* canopy biomass from 1978 to 1983 at all three islands (Fig. 7). Kelp recruited in 1983, but with the thermal stress of the El Niño, canopy formation was not significant until 1984. Thus, the very slow abalone growth observed during 1983 to 1984 can be attributed to thermal stress (Fig. 5), low kelp biomass (Fig. 6, Fig. 7), and the low drift production of a young *Macrocystis* stand (Tegner & Dayton 1991).

Statewide commercial red abalone landings began a significant

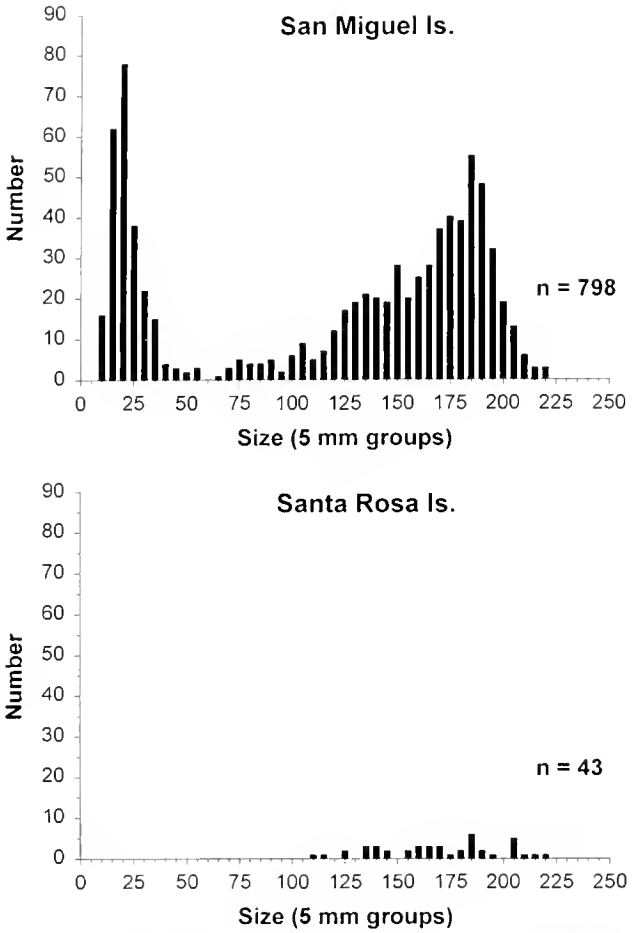

Figure 8. Red abalone size-frequency distribntions from San Mignel and Santa Rosa Island, June 1999.

decline in the late 1960s (Fig. 9). When commercial fishing was closed in 1997, total red abalone landings were at 87 metric t, almost a 90% decline from the historic peak in the early 1960s. Karpov et al. (2000), analyzing area-specific landings, showed a spatial trend with higher catches coming from the mainland or nearshore islands, shifting over time to more remote areas. Catch from Santa Cruz Island, the closest to shore, peaked before Santa Rosa Island; at the time of the closure, catch on the two islands was 1 and 3% of their respective peaks. In contrast, San Miguel Island was the source of 82% of the red abalones landed in 1996, and these represented 23% of the peak catch on this island. Fishery-independent abundance estimates collected from 1983 to 1998 confirmed that populations declined in conjunction with the catch (Fig. 9). Red abalone population estimates in Santa Cruz Island study sites (see Davis 1989 for site locations and survey techniques) went to zero in the late 1980s; Santa Rosa Island had higher initial abundance and density estimates that also declined to low levels by the end of the 1990s. Abundance estimates at San Miguel Island mirrored the continuing catch until the fishery was closed in 1997 (Karpov et al. 2000).

The temperature, kelp, and abalone reproduction and recruitment data suggest that environmental variability contributed to the decline of the fishery and the subsequent collapse of some stocks. If, as Cox (1962) reported, there was little to no recruitment of red abalones during 1957 to 1959, there would have been a corresponding decline of animals entering the fishery 10 to 15 years later. The decline in statewide landings and in the Santa Cruz and Santa Rosa Island catches in fact began about 1968 (Fig. 9). Under intense fishing pressure, few abalones exceed commercial size limits in natural populations (Tegner et al. 1989) and aggregations are fewer and smaller (Shepherd & Partington 1995). As the reduced or missing age classes passed through the size-frequency distribution to fishable size, stocks would experience a corresponding decrease in density and egg production. While stock-recruitment relations are poorly known for abalones, it appears that major environmental disturbances such as the 1957 to 1959 El Niño coupled with continued intense fishing pressure establish the conditions for recruitment overfishing. Shepherd and Partington (1995) report that below densities of 0.15 to 0.2 m^{-2}, abalone populations are increasingly vulnerable to recruitment failure. Fishery independent data (Fig. 9) indicate that the Santa Cruz Island red abalone population was below this threshold in 1983, at the beginning of a strong El Niño event, and these animals disappeared from the study sites within four years. At Santa Rosa Island, the 1983 abundance estimate was 0.15 m^{-2}, but this study population did not survive the environmental disturbances of following years. Fig. 8 indicates that there are still red abalones on Santa Rosa Island, but the lack of recruitment during conditions in late 1998 through 1999 which produced strong recruitment at San Miguel Island suggests that Santa Rosa Island densities are below the threshold for successful fertilization.

Once all three of these northern Channel Islands were highly productive red abalone habitat (Karpov et al. 2000). San Miguel Island populations have persisted not only because it is the furthest of the three islands from ports, but because its ocean climate has apparently buffered recent environmental variability relative to the warmer islands. Indeed, fishermen find it easier to get to San Miguel Island than Santa Rosa Island by following the mainland coast to Point Conception before crossing the Santa Barbara Channel (J. Colgate pers. comm.). The role of climate in exacerbating overfishing is supported by catch statistics from other locations. Red abalone catch from the San Diego region, apparently heavily impacted by the 1982 to 1984 El Niño (Tegner & Dayton 1987), dwindled to very low levels in the late 1980s, whereas Point Conception stocks were still productive when the fishery was closed in 1997 (California Department of Fish and Game 1997).

One of the most disturbing implications of El Niño events and interdecadal scale increase in temperatures for abalones involves Withering Syndrome (WS), a disease first identified in black abalones (*H. cracherodii*) and now observed in other California species as well. Elevated temperatures accelerate mortality rates (Friedman et al. 1997), and Tissot (1995) suggests that temperature is the single most important factor influencing population recovery. WS is now progressing through Central California (Altstatt et al. 1996). During the elevated temperatures of the 1997 to 1998 El Niño, several abalone farms in California experienced a dramatic increase in the proportion of red abalones showing clinical signs of WS (Moore et al. 2000).

Management of California abalone fisheries was heavily based on the high fecundity of these gastropods; a size limit large enough that the animals would reproduce several times before entering the fishery was considered adequate to protect the stocks. Later egg-per-recruit analysis indicated that the size limits allowed for healthy egg production, even at high fishing mortality rates (Tegner et al. 1989). The implicit assumptions of the size-limit approach, however, didn't consider extended periods of environmen-

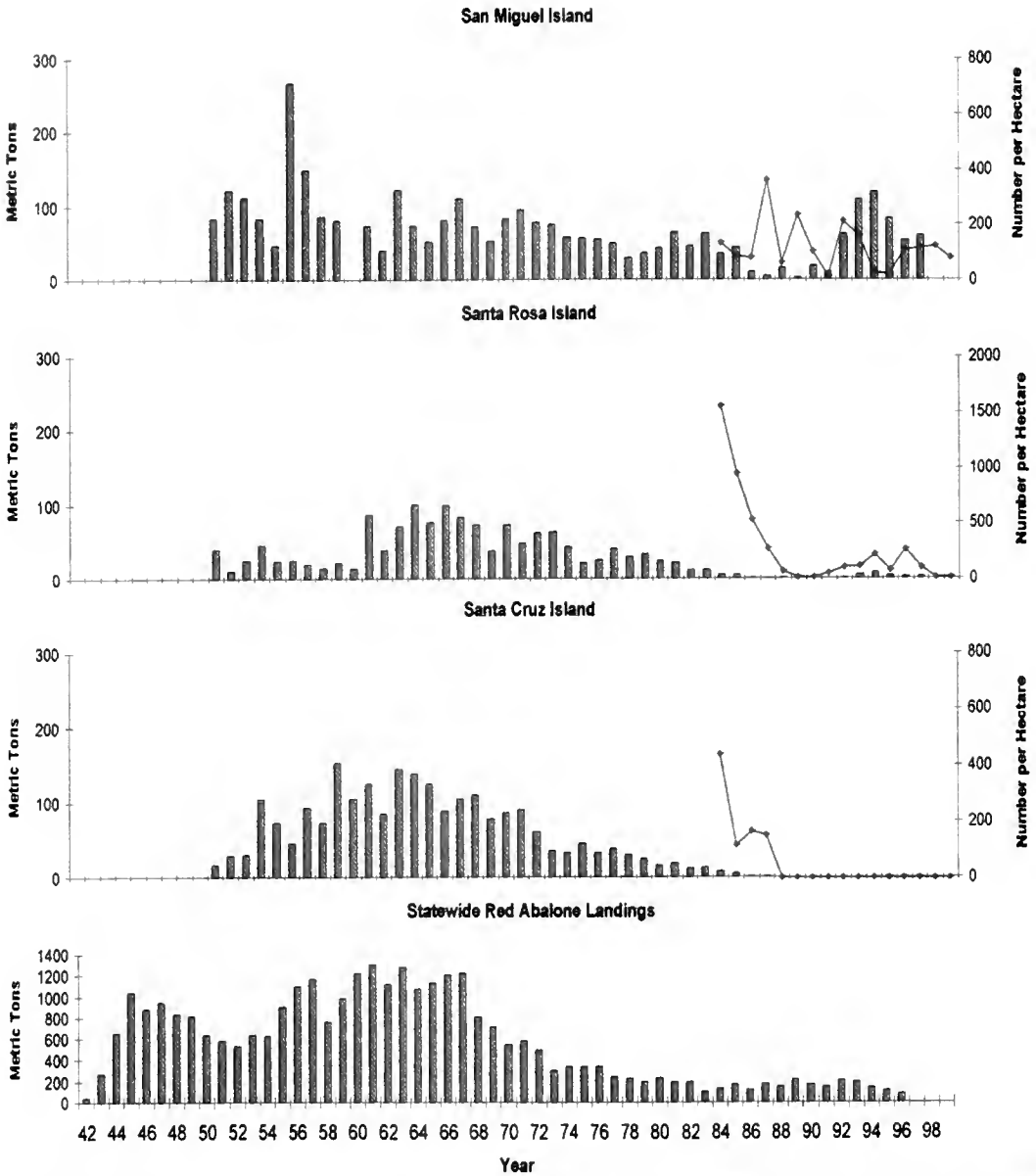

Figure 9. Red abalone harvest (metric tons) from San Miguel, Santa Rosa, and Santa Cruz Island, 1952 to 1996, and non-invasive population counts on 60 m^2 transects from the Channel Islands National Park annual monitoring program, 1983 to 1998. Statewide landings, 1942 to 1996. Note the change in scale on the vertical axis. Adapted from Karpov et al. (2000).

tal variability leading to protracted recruitment failure or Allee effects on fertilization (Shepherd & Partington 1995). The organisms of west coast kelp communities are adapted to environmental variability on scales including episodic El Niño and La Niña events, and interdecadal regime shifts. The Scripps pier temperature record reveals three regimes since the beginning of the data set in 1916: a warm period until about 1940, a cool period lasting until 1976, followed by the present warm period (McCall 1996). Red abalone reproduction and growth may be depressed during periods of warm water, but most animals survive the adverse environmental conditions; the addition of intense fishing pressure onto climate disturbances, however, makes population decline almost inevitable. To be successful, future management must take both environmental variability and Allee effects into account; no-take reserves to protect aggregations of brood stock are probably the only way to reduce Allee effects. Our data suggest that remote monitoring of temperature and kelp canopy biomass could be calibrated to offer an inexpensive and practical approach to incorporate environmental variability into management of abalones and other grazers.

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POPULATION GENETICS OF THE YELLOW ABALONE, *HALIOTIS CORRUGATA*, IN CEDROS AND SAN BENITO ISLANDS: A PRELIMINARY SURVEY

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ABSTRACT The yellow abalone, *Haliotis corrugata*, is one of the principal species caught in Central Baja California, Mexico. Around the Cedros and San Benito Islands, the yellow abalone is mainly distributed in three zones: the North (Punta Norte) and South (San Agustin) of Cedros Island and around the small islands of San Benito. The main goal of this work was to characterize genetically the populations of the yellow abalone in these three zones as a baseline to help the fishery. Allozyme electrophoresis at eight loci was carried out with six samples from two years in the three localities. The average number of alleles per locus was 2.3 with a 67% polymorphism. Mean unbiased heterozygosity ranged from 0.15 to 0.25, which is similar to that of other abalone species. Only three out of 34 cases did not agree with the Hardy-Weinberg equilibrium and there was no tendency for either heterozygote excess or deficit. It was concluded that *H. corrugata* shows differentiation between localities and these may be considered as independent populations for fishery management.

KEY WORDS: abalone, *Haliotis corrugata*, population genetics, allozymes, Cedros Island, San Benito Islands

INTRODUCTION

In Mexico, *Haliotis corrugata* is known as the yellow abalone because of the color of its foot. It is distributed from the Coronado Islands near the border with the United States to Margarita Island, and in depths from 10 to 20 m (Guzmán del Próo 1992). The yellow abalone together with the blue abalone, *Haliotis fulgens*, are the principal products from the Mexican fishery with annual production about 709 t (meat weight) in 1998 (SEMARNAP 1999). Along the Peninsula of Baja California, *H. corrugata* represents about 13% of the fishery's production (León & Ramírez 1992).

The Cooperative "Pescadores Nacionales de Abulón" (PNA) capture abalone from the Cedros and San Benito Islands (Fig. 1) and the annual production in the past 11 y is shown in Table 1. In these islands, abalone is mainly distributed in three zones: (a) the North and (b) South of Cedros Island, and (c) around the small islands of San Benito.

There are few studies on abalone population genetics on the Pacific Coast of North America (Gaffney et al. 1996, Kirby et al. 1998, Gutiérrez-González 2000, Hamm & Burton 2000, Zúñiga et al. 2000), but to our knowledge there are no studies on population genetics of the yellow abalone. Therefore, the aim of this study was to carry out a preliminary genetic characterization of the yellow abalone, *Haliotis corrugata*, population at the Cedros and San Benito Islands using allozyme electrophoresis, to determine the population genetic structure and to obtain initial genetic data for fishery management and for population enhancement.

MATERIALS AND METHODS

Adults of *H. corrugata* were sampled from the commercial catch and from a population evaluation program. These abalone were caught from six localities: "El Faro," "La Bandera," "Anegados," and "El Chual" during 1997, and "Punta Norte" and "San Agustin" during 1998 (Fig. 1). Their shell length was measured and locality mean shell lengths were compared with a one-way ANOVA, followed by an *a posteriori* Tukey test. Gill and digestive gland samples were individually frozen in 1.5-ml tubes and transported to the Laboratory of Genetics at Centro de Investigación Científica y de Educación Superior de Ensenada, B.C. (CICESE) and preserved at −70 °C until electrophoresis analysis. Abalone tissues were homogenized individually with 0.5 ml of TME buffer (0.1 M Tris, 0.1 M maleic acid, 0.01 EDTA, and 0.01 $MgCl_2$, pH 7.8), and allozyme electrophoresis on 12% starch gels was carried out for 11 enzymatic systems: aspartate aminotransferase (AAT, 2.6.1.1), glucose phosphate isomerase (GPI, 5.3.1.9), glutathione reductase (GSR, 1.6.4.2), hexokinase (HK 2.7.1.1), isocitrate dehydrogenase (IDH, 1.1.1.42), leucine amino peptidase (LAP, 3.4.11. −), malic enzyme (ME 1.1.1.40), malate dehydrogenase (MDH 1.1.1.37), mannose-phosphate isomerase (MPI, 5.3.1.8), 6-phospho-gluconic dehydrogenase (PGD, 1.1.1.44), and phospho-glucomutase (PGM, 2.7.5.1). Gels were run in the TME buffer (pH 7.8) and staining was performed using standard procedures (Harris & Hopkinson 1976), except for LAP which was stained according to procedures described by Beaumont et al. (1983). Meldola blue (0.8%) was used instead of PMS (Turner & Hopkinson 1979). When more than one locus was observed, the most cathodical locus was named as '1.' The most common allele was designated as "100" and the rest were designated according to their relative mobility to the 100 allele.

Allele frequencies, polymorphism, unbiased and direct count heterozygosity, heterozygote deficiency (D), and Nei's (1978) genetic distances were evaluated using the Biosys-1 (Swofford & Selander 1981), and an unweighed pair group method with arithmetic means (UPGMA) phenogram was built. Deviations from the agreement with the Hardy-Weinberg (H-W) equilibrium were tested with the Genepop's exact test (Raymond & Rousset 1994). Wright's (1951, 1965) F (F_{IS}, F_{ST}, and F_{IT}) statistics were evaluated using Weir and Cockerham's (1984) estimators by jacknifing and their significances were tested by bootstrapping with the Fstat program (Goudet 1994). Pairwise comparisons among all populations were done using exact tests (Miller 1997). Whenever there

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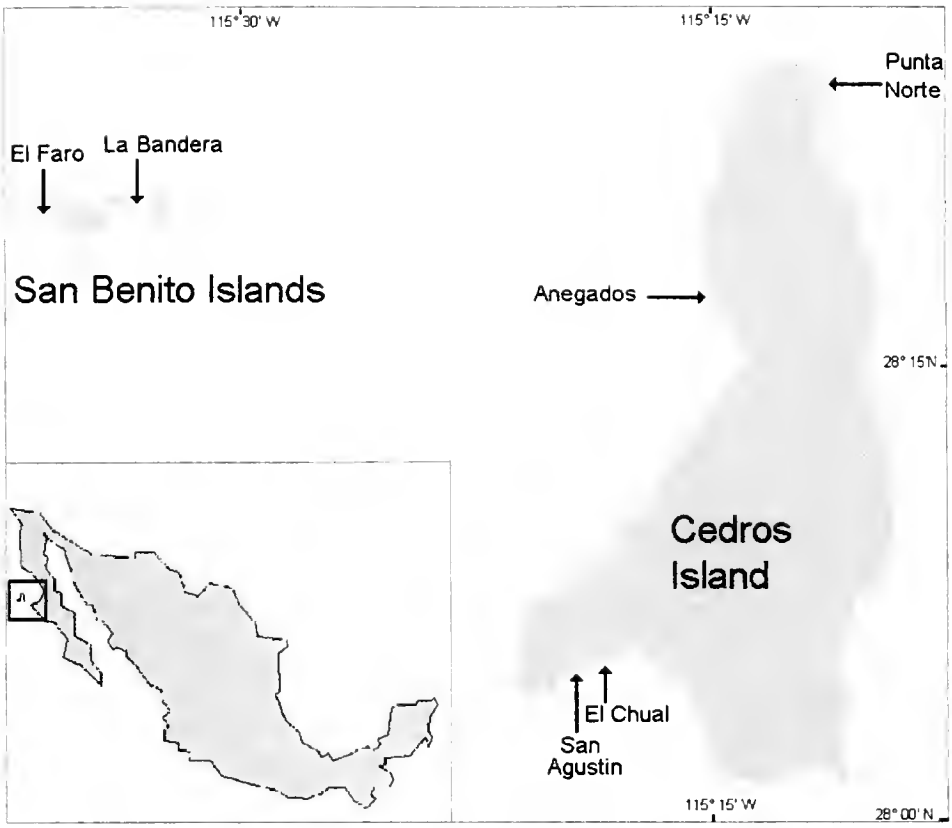

Figure 1. ***Haliotis corrugata*** **sampling localities in Cedros and San Benito Islands.**

was a high number of tests, the level of significance (α) was Bonferroni adjusted using the Dunn-Šidák method [$\alpha'' = (1 - (1 - 0.05)^{1/k})$; where k = number of test] (Sokal & Rohlf 1995).

RESULTS

Yellow abalone mean sizes were between 128 and 157 mm; there were significant differences between localities, but four out of six localities had similar sizes (Table 2).

From the 11 allozyme systems tested, only eight loci gave reliable results (Table 2). PGD, GSR, and MPI did not give positive staining results. Two loci were found at LAP and MDH, but the first locus of each one was not reliable and thus, excluded from further analysis.

The mean sample size was between 15.4 to 31.8 and the number of alleles per locus was from 1.9 to 2.8. The percentage of polymorphism without criterion ranged from 62.5% to 87.5%, while direct count frequency of heterozygosity was from 0.094 to 0.201 and unbiased frequency of heterozygosity was from 0.141 to 0.258. In all cases El Chual had the lowest values (Table 2). Frequencies of the 100 allele were similar between localities except at the *Hk* in Punta Norte and at *Mdh-2* in La Bandera, which both showed substantially reduced frequencies of the most common allele (Table 3).

Agreement with H-W was present in all cases, except in 3 loci from Punta Norte (after Bonferroni adjustment), all of which had a heterozygote deficiency (Table 3). Furthermore, all positive and negative cases of *D* were counted, the total was $D+ = 21$ and $D- = 13$, which was not significant from a 1:1 proportion (sign test, Sokal & Rohlf 1995).

The UPGMA phenogram using Nei's genetic distance grouped localities: first La Bandera with El Chual, then El Faro with Anegados, and then these two groups clustered together, then San Agustin joined and finally Punta Norte (Fig. 2).

Wright's F statistics showed positive and negative values for the loci (Table 4). F_{IT} had three positive values significantly different from zero at the *Lap2*, *Mdh2*, and *Hk* loci (Table 4). Four cases of F_{ST} were significantly different from zero at the *Lap2*, *Me*, *Mdh2*, and *Hk* loci. While the F_{IS} had two negative and significant values at *Me* and *Aat* and only one negative and non-significant value at the *Pgm* locus. All mean F values were significantly

TABLE 1.

Haliotis corrugata **production from the Cooperative "Pescadores Nacionales de Abulón" at Cedros and San Benito Islands.**

Year	Meat weight (kg)	Percentage from total[1]
1989	8,241	8.31%
1990	12,930	7.87%
1991	10,047	5.17%
1992	6,542	3.46%
1993	—[a]	—
1994	2,384	1.26%
1995	23,807	12.67%
1996	23,912	12.05%
1997	23,174	12.84%
1998	24,698	18.57%
1999	16,612	24.13%
2000		

Source Alejo Ojeda Ibarra, bookkeeper S.C.P.P. "Pescadores Nacionales de Abulón" S.C. de R.L.

[a] There was no capture of yellow abalone in 1993.

TABLE 2.

Mean shell length, mean sample size per locus (n), mean number of alleles per locus (al), percentage of loci polymorphic (%, without criterion) and heterozygosity (dc = direct count; and Nei's unbiassed H) of *Haliotis corrugata* in Cedros and San Benito Islands (SE).

	Population					
	1997				1998	
Locus	El Faro	La Bandera	Anegados	El Chual	San Agustin	Punta Norte
Shell length*	153.53[a]	128.75[c]	138.11[b]	156.70[a]	154.07[a]	156.00[a]
	(1.80)	(2.15)	(1.84)	(2.49)	(1.80)	(2.52)
n	22.6	15.5	24.4	15.4	31.8	22.9
	(2.2)	(1.3)	(2.2)	(1.1)	(1.4)	(2.4)
al	2.3	2.1	2.8	1.9	2.3	2.3
	(0.3)	(0.4)	(0.5)	(0.3)	(0.3)	(0.4)
Polymorphism**	87.5	62.5	75.0	62.5	87.5	75.0
H d.c.	0.201	0.196	0.139	0.094	0.193	0.118
	(0.076)	(0.069)	(0.039)	(0.033)	(0.040)	(0.031)
H	0.174	0.200	0.166	0.141	0.203	0.258
	(0.059)	(0.071)	(0.049)	(0.063)	(0.043)	(0.075)

* Superscripts show statistically equal mean shell lengths.

** A locus was considered polymorphic if more than one allele was detected.

different from zero, indicating heterozygote deficiency and population differentiation. A pairwise comparison among populations was carried out and it did not show differentiation between La Bandera, El Chual and Anegados after Bonferroni adjustment (Table 5). These differences were mainly produced by *Mdh2* and *Hk* loci (Table 5).

DISCUSSION

This is a preliminary survey where the analysis of 8 allozyme loci shows genetic differences between six localities of the yellow abalone *Haliotis corrugata* in Cedros and San Benito Islands, B.C. Mexico. The number of loci sampled is approximately the mean of the numbers of other tested loci in different abalone species (Table 6). Hamm and Burton (2000) found genetic differentiation between localities of the black abalone, *Haliotis cracherodii,* using three allozyme loci (*Aat-1, Pgi* [= *Gpi*], and *Pgm*), although comparisons with partial DNA sequences of the mtDNA encoded cytochrome oxidase subunit I gene failed to differentiate between populations. These loci did not differentiate between three populations of the red abalone, *Haliotis rufescens* (Burton & Tegner 2000). Similarly, 7 loci tested with the blue abalone, *Haliotis fulgens,* failed to separate six localities in the middle of the Peninsula of Baja California (Zúñiga et al. 2000), and 16 loci did not separate four populations between Isla de Cedros and Isla Magdalena (Gutiérrez-González 2000).

Size difference among samples is not considered to have an influence in population differentiation because localities with similar abalone size did not join together (Fig. 2, Table 2). Sample sizes are similar to those from the blue abalone (Zúñiga et al. 2000) and may be considered as small sample size, but the statistical methods used here are considered to be robust enough to avoid the bias produced by small sample size.

The polymorphism, mean allele number per locus, and the observed heterozygosity found in *H. corrugata* are similar to other abalone species, marine invertebrates and invertebrates in general (Table 6).

Only Punta Norte population was not in H-W equilibrium and was genetically more separated from the other populations, which may be due to its location on the East coast of Cedros Island. In this area, an anticyclonic eddy is formed during August and September due to an upwelling event on the coast of the Peninsula of Baja California (Mancilla-Peraza et al. 1993, Palacios-Hernández et al. 1993, Amador-Buenrostro et al. 1995). The main stream east of the Cedros Island goes north around the island and then west towards San Benito Islands, and from these to the San Agustin area (Palacios-Hernández et al. 1993). The spawning peaks in these areas are in August and in November, then, if spawning occurs when currents go north-south to the west of Cedros Island, currents could transport some larvae from one place to another; these larvae could be carried from Anegados and San Benito towards the south of Cedros Island. There are no studies regarding abalone larval dispersal and oceanographic conditions around Cedros and San Benito islands during spawning time. However, it is considered that migration is small among abalone populations (Guzmán del Próo et al. 2000). Although, Guzmán del Próo et al. (2000) consider that abalone larvae usually settle around parental beds, in some cases, larvae may be transported about 3–5 km; because this distance is smaller than the actual distance among islands, it is considered that geographical distance may be one of the most important factors in population differentiation and in H-W equilibrium of *H. corrugata.*

Other factos may influence the agreement with H-W equilibrium (i.e. selection, migration, and inbreeding). Fishing may be considered as a selection pressure, because abalone larger than 140 mm are commercially caught. Environmental factors may have an effect on the population structure. It has been observed that sea surface temperature anomalies have a positive effect on *H. corrugata* population (Shepherd et al. 1998). This may reduce stock density and the possibility of genetic differentiation due to genetic drift increases.

Non-random mating may be considered to be important in population differentiation because of the spawning behavior of abalone and because larvae tend to settle in adult beds (Guzmán del Próo et al. 2000). Another source of non-random mating is the introduction of cultured organisms and may have an influence on

TABLE 3.

***Haliotis corrugata*. Allele frequencies and heterozygote deficiencies (*D*) and their significance after α adjustment.**

	Population					
	1997				1998	
Locus	El Faro	La Bandera	Anegados	El Chnal	San Agustin	Punta Norte
Aat						
92	0.019	0.000	0.000	0.000	0.000	0.000
100	0.926	0.900	1.000	1.000	0.939	1.000
140	0.056	0.100	0.000	0.000	0.061	0.000
D	0.044	0.074			0.048	
Gpi						
85	0.089	0.075	0.145	0.026	0.135	0.100
90	0.018	0.000	0.000	0.000	0.000	0.000
100	0.893	0.900	0.855	0.974	0.851	0.900
110	0.000	0.025	0.000	0.000	0.014	0.000
D	0.082	0.061	−0.361	0.000	−0.065	0.093
Hk						
91	0.000	0.000	0.000	0.000	0.000	0.429
100	0.938	1.000	1.000	1.000	0.870	0.571
106	0.063	0.000	0.000	0.000	0.130	0.000
D	0.044				0.128	−0.620*
Idh						
87	0.000	0.000	0.067	0.000	0.000	0.000
91	0.000	0.056	0.050	0.000	0.000	0.000
100	0.957	0.861	0.833	0.941	0.939	0.904
104	0.000	0.028	0.017	0.000	0.000	0.000
107	0.000	0.000	0.000	0.000	0.000	0.038
112	0.043	0.056	0.033	0.059	0.061	0.058
D	0.023	0.074	−0.228	0.031	0.048	0.058
Lap-2						
90	0.000	0.000	0.000	0.036	0.000	0.000
100	1.000	1.000	0.925	0.929	0.780	0.682
104	0.000	0.000	0.025	0.000	0.220	0.318
110	0.000	0.000	0.025	0.000	0.000	0.000
120	0.000	0.000	0.025	0.036	0.000	0.000
D			0.026	0.019	−0.200	−0.800*
Mdh2						
100	0.630	0.500	0.780	0.647	0.891	0.600
105	0.217	0.000	0.000	0.000	0.078	0.033
110	0.000	0.000	0.020	0.000	0.000	0.000
120	0.152	0.500	0.200	0.353	0.031	0.367
D	0.279	−0.352	−0.219	−0.500	−0.230	−0.872*
Me						
80	0.132	0.000	0.000	0.000	0.000	0.000
92	0.000	0.000	0.024	0.000	0.000	0.000
100	0.868	1.000	0.976	1.000	1.000	1.000
D	0.121		0.000			
Pgm						
89	0.000	0.056	0.017	0.150	0.000	0.033
100	0.964	0.722	0.879	0.800	0.792	0.883
109	0.036	0.222	0.052	0.050	0.153	0.050
115	0.000	0.000	0.052	0.000	0.056	0.033
D	0.019	0.232	0.073	−0.148	0.027	−0.085

* Significant deviation from H-W after α adjustment.

the genetic structure of a population, by reducing the genetic pool (Gaffney et al. 1996).

With the mean value of F_{ST} 0.093, it can be considered that the localities of *H. corrugata* conform to discrete populations with moderate diversification, since this value is between 0.05 and 0.15 (Wright 1951, Wright 1965). This contrasts with the low diversification of *H. fulgens* in Mexico which shows low values of F_{ST}, 0.022 to 0.036 (Table 6), and it has been considered that *H. fulgens* forms a single population from Cedros Island to Isla Magdalena (Gutiérrez-González 2000). *H. corrugata* also shows a

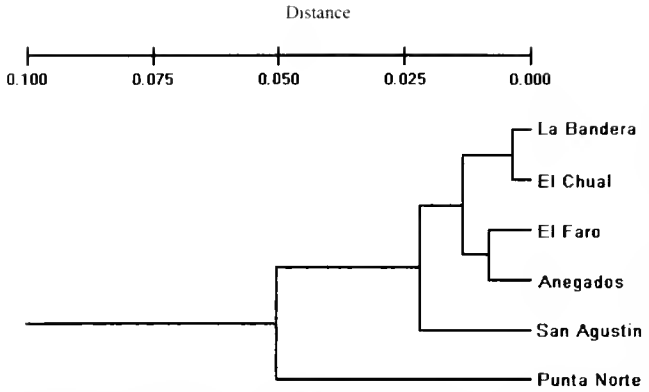

Figure 2. Nei's (1978) unbiased genetic distance for the yellow abalone, *Haliotis corrugata*, at six localities on Cedros and San Benito Islands. Cophenetic correlation = 0.904.

TABLE 4.

***Haliotis corrugata* F statistics.**

Locus	F_{IT}	F_{ST}	F_{IS}
Aat	−0.044	0.008	−0.052*
Gpi	0.074	−0.004	0.078
Hk	0.715*	0.360*	0.411
Idh	0.072	0.007	0.065
Lap2	0.436*	0.129*	0.333
Mdh2	0.336*	0.110*	0.225*
Me	−0.004	0.154*	−0.171*
Pgm	0.037	0.038	−0.001
Mean	0.240*	0.093*	0.160*

* Significantly different from 0 after α adjustment.

TABLE 5.

Probability values (below the diagonal) obtained after pairwise comparisons among all populations using exact tests.[1] Loci where there was a difference between populations (above the diagonal) after α adjustment.[2]

Population	1	2	3	4	5	6
1 El Faro	***	*Mdh2*	*Mdh2*	*Pgm*		*Hk*
2 La Bandera	0.0007	***			*Mdh2*	*Hk*
3 Anegados	0.0034	0.1133	***			*Lap2, Hk*
4 El Chual	0.0014	0.6875	0.2752	***	*Mdh2, Pgm*	*Lap2, Hk*
5 San Agustin	0.0006	<0.0001	0.0001	<0.0001	***	*Mdh2, Hk*
6 Punta Norte	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	***

[1] 10,000 dememorization steps, 30 batches and 10,000 permutations per batch.
[2] $\alpha'' = 1 - (1 - 0.05)^{1/15} = 0.00341$.

TABLE 6.

Genetic parameter comparison between different abalone species.

	No. loci	No. sites	*n*	Ho	Polymorphism (%)	Mean allele number per locus	F_{IT}	F_{ST}	F_{IS}	Reference
H. cracherodii	3	7	~427					0.039	0.125	Hamm & Burton 2000
H. fulgens				0.067						Brown 1993
H. fulgens	7	5	102	0.054–0.195	14.3–100.0	1.7–2.0	0.335	0.036	0.318	Zúñiga et al. 2000
H. fulgens	12	4	377	0.061–0.075	38–44	1.96–2.06	0.637*	0.022	0.629*	Gutiérrez González 2000
H. laevigata	15	8		0.195	73.3	2.67	0.062	0.014	0.076	Brown & Murray 1992
H. rubra	15	15		0.140	63.1	2.58	0.056	0.016*	0.071	Brown & Murray 1992
H. rubra								0.022		Brown 1991
H. rufescens	3	3	131					0.012		Burton & Tegner 2000
H. rufescens[1]	13	7[2]	714	0.056–0.291	38.46–75	1.5–2.1				Licona-Chávez 1999
H. tuberculata				0.284		2.3–3.0				Mgaya et al. 1995
H. virginea				0.125						Brown 1993
H. corrugata	8	6	183	0.094–0.201	62.5–87.5	1.9–2.8	0.240*	0.093*	0.160*	This work
Marine invertebrates				0.061–0.216						Fujino et al. 1983
Invertebrates				0.101						Nevo 1978

* Significantly different from zero.
[1] Cultivated.
[2] Number of batches.

slightly higher F_{ST} value in comparison with other abalone species, showing more diversification (Table 6).

Yellow abalone localities at Cedros and San Benito Islands form different populations, which should be considered in fishery management. More studies on other populations of the yellow abalone need to be performed to test whether population differentiation is also present along the Peninsula of Baja California. Also, studies on larval ecology should be carried out to determine migration among islands.

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MOVEMENT AND RE-AGGREGATION OF THE BLACKLIP ABALONE, *HALIOTIS RUBRA* LEACH, AFTER FISHING

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ABSTRACT Movement and re-aggregation after fishing of a stock may have a major impact on estimates of abundance and stock assessment parameters such as natural mortality. The propensity of blacklip abalone (*Haliotis rubra* Leach) to re-aggregate after fishing was studied using a combination of *in situ* tagging with fine-scale mapping of two distinctly different abalone populations. Controlled fishing of each population was conducted to test the hypothesis that removal of abalone by fishing stimulates movements that result in re-aggregation. Declines in abundance due to fishing were evident but less than those expected had no recovery occurred. Changes in the fine-scale spatial distribution of abalone suggested that re-aggregation occurred through a series of contiguous displacements. Smaller emerging abalone did not appear to be the prime source for this re-aggregation. These results undermine the utility of change-in-ratio and catch-per-unit-effort methods of abundance estimation. They also highlight the need to develop methods of abundance estimation that accommodate the impact of aggregating behavior on blacklip abalone populations.

KEY WORDS: *Haliotis rubra*, abalone, movement, aggregation

INTRODUCTION

Blacklip abalone, *Haliotis rubra*, form the basis of a large and extremely important fishery in southern Australian waters. The year 2000 total allowable catch of 2720 tons was worth about $AUD 80 million at the point of first sale. Despite the economic importance of abalone fisheries, stock assessment of abalone has remained problematic (Breen 1992). These difficulties are partly a product of the biology of abalone and the way they are fished. The tendency of abalone to live in aggregations renders them more vulnerable to overfishing by increasing their catch rates (McShane 1995). Divers are able to maintain high catch rates by moving from aggregation to aggregation and by relocating to a different area once catch rates drop below an acceptable level (Prince 1989, Breen 1992, McShane 1995). If the abalone remaining after fishing then re-aggregate, a diver returning to the area may well fish the area at a similar catch rate to the first excursion. These processes may all contribute to hyper-stability in catch rates and conceal real fluctuations in population size (Breen 1992, Prince & Shepherd 1992, McShane 1994).

Understanding how abalone reform aggregations is therefore central to understanding the resilience of abalone populations to fishing. Furthermore, because stock assessment techniques are based on assumptions about the spatial and temporal distribution of animals, understanding the nature of re-aggregation is crucial to the choice of tools used for stock assessment.

This paper first describes the response of two blacklip abalone populations to experimental fishing. It then focuses on determining where "replacement" abalone come from to reform aggregations and how these processes change with time. Several hypotheses that could explain re-aggregation are examined. These include emergence (from cryptic habitat within the fished area), immigration (from outside the fished area), relocation (from sparsely populated regions within the fished area), and a combination of these processes. A fifth hypothesis of growth (of pre-recruits to recruits) is not examined because growth was negligible during the period of the study.

MATERIALS AND METHODS

Experimental sites were selected off the coast of Victoria, Australia, at Point Cook and at Flinders (Fig. 1). Both sites were subject to little illegal or recreational abalone fishing, and were closed to commercial fishing for six months prior to and throughout the experiment. The sites were chosen to represent extremes of habitat type and abalone distribution.

The Point Cook site (37°55.893′ S, 144°47.104′ E) was 3–4 m in depth and was not subject to strong swell or current. The reef consisted of basalt boulders, rarely over 1 m in height, on a sandy substrate. Conditions appeared anaerobic beneath the boulders and there was very little cryptic habitat that could not be thoroughly searched by a diver. The dominant macro-algae included *Ecklonia radiata*, *Caulerpa* spp., *Enteromorpha linza*, *Cystoseria* spp., and, during summer months, a thick mat of drift red algae (mostly *Jeanerettia pedicellata*). Abalone were abundant at Point Cook and were relatively evenly distributed. At Flinders (38°29.397′ S, 145°01.274′ E) the depth was 7 to 9 m and the site was prone to strong swells and currents. The reef consisted of fractured and stepped basalt platforms, often over 1 m in height, and interspersed by sand gutters. The fractured nature of the reef provided an abundance of unsearchable cryptic habitat. The dominant algae included *Phyllospora* spp., *Ecklonia radiata*, and coralline algae. Abalone were less abundant at Flinders than at Point Cook and were more patchily distributed.

Four square plots were set up in fixed positions at each site. The boundaries of each plot measured 24 m and each plot was separated from the other plots by 10-m corridors (Fig. 2). At each site two plots were fished to assess the impact of fishing and two plots were left unfished to compare the fishing impact with unfished areas. Fished and control plots were chosen randomly, but were constrained to being diagonally opposed. Surveys were conducted prior to fishing, 3–4 wk after fishing (to assess the impact of fishing), and, 10 wk later to assess the recovery from fishing. This design allowed for temporal and spatial comparisons between plots, both fished and control, and between sites.

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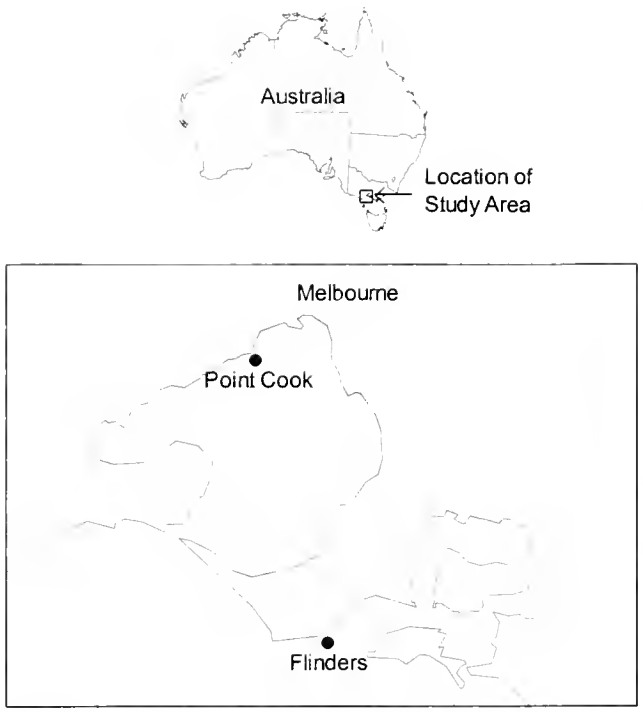

Figure 1. Location of the experimental sites at Point Cook and Flinders.

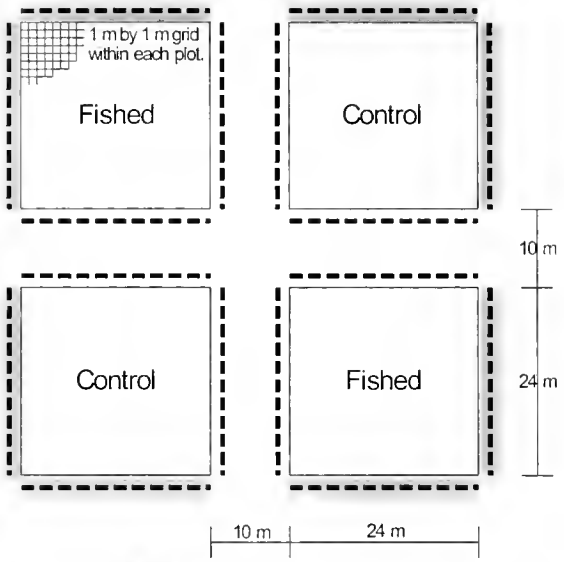

Figure 2. Diagram showing the set-up of experimental areas at each site. Shaded areas outside the plots indicate areas in which abalone were mass marked. Dotted lines indicate the position of fixed transects swum outside the plots.

Changes in abundance were assessed by counting all emergent abalone within the plots using an exhaustive 1 × 1 m grid system. These grid counts were also used to calculate Morisita's index of dispersion (Krebs 1989) (Eq. 1):

$$I_d = n\left[\frac{\sum x^2 - \sum x}{(\sum x)^2 - \sum x}\right]$$

where I_d is Morisita's index of dispersion, n is the sample size, $\sum x$ is the sum of grid counts, and $\sum x^2$ is the sum of grid counts squared.

This index is a useful descriptor of the degree of aggregation in observed spatial patterns. The significance of observed spatial patterns was tested using the standardized Morisita index of dispersion (described in Krebs 1989). This index allows comparisons between plots and sites because it is independent of both population density and sample size.

Differences in the spatial distribution of abalone were also studied by measuring the distance between abalone and their first, second, and third nearest neighbors. The source individuals for these measurements were selected randomly from cells with the greatest and least abundance of abalone within each plot. A chi-square goodness-of-fit test was also used to compare the observed and expected distributions of nearest-neighbor distances (Campbell & Clarke 1971). These tests indicated the significance of any departure from random spacing. The shape of the observed distributions indicated any departure from a random pattern. The distribution of nearest-neighbor distances at each survey was compared to assess temporal changes in the degree of aggregation.

During each survey the abundance of abalone outside each plot was estimated by making counts along fixed transects (Fig. 2). These transects measured 24 m in length by 1 m in width. These counts were made to assess the extent of immigration into the plots.

Lengths were recorded to assess fishing-induced changes in the size composition of the population. The extent of weed growth on shells was also noted. These data were collected under the assumption that abalone emerging from cryptic habitat were smaller and carried less algal cover than the conspicuous population.

To examine immigration and relocation abalone were individually tagged. This was done with a rivet tag through a respiratory pore using a method modified from that of Prince (1991). All abalone were tagged and measured *in situ* to minimize disturbance and displacement. To spread the tagging within the plots across the range of conspicuous abalone sizes and across all habitats, divers attempted to tag every fifth abalone sighted. The positions of tagged abalone were then recorded within the grid to an accuracy of ±0.1 m. The 1322 and 819 abalone tagged within the plots at Point Cook and Flinders represented 19 and 18 percent of the unconcealed populations, respectively.

To assess the extent of immigration into the plots after fishing, abalone on the outer perimeters of the plots were also marked (Fig. 2). Starting closest to each plot boundary and working outwards, all abalone encountered were marked until at least 170 had been either tagged or painted with a uniquely colored paint-stick (50% for each marking method). Abalone between the inner borders of the plots were not marked so that interaction effects along the internal borders between plots were avoided. Approximately 1400 abalone were marked outside the plots at each site.

The relocation of tagged abalone within the plots was examined by using grid-cell abundance as an index of habitat quality. The assumption was that the greater the number of abalone initially resident within a grid cell, the better the habitat quality of that cell. The initial abundance in cells that tagged abalone occupied prior to moving was compared with that of the cells in which they settled. Abalone were determined to have settled when subsequent surveys revealed that they had moved less than one meter from their previous position.

Experimental fishing was done above minimum size limits. The size limits used were less than those normally in use in the Victorian abalone fishery (Point Cook: normally 100 mm, used 93 mm; Flinders: normally 110 mm, used 100 mm). These size limits made a greater proportion of the population available to fishing and hence, allowed a greater fishing impact. About 50% of the population at each site were over the respective size limits. Of those, 20% were tagged and therefore unavailable to fishing. The remaining 40% of the population oversize were fished until a reduction of 35% of the abundance prior to fishing was achieved. To allow comparison between sites the same relative reduction in abundance was applied at both sites.

RESULTS

Changes in Abundance

Changes in the total abundance of abalone counted within the plots were compared as a percentage of the initial abundance counted prior to fishing (Fig. 3). At each site changes in abundance were consistent within each type of plot and were therefore combined for analysis. In all cases the observed change in abundance was different to that expected. If no movement had occurred after fishing, a 35% reduction was expected in fished plots, and no change was expected in control plots. At both sites a decrease in abundance of less than 20% was observed in fished plots. An increase in abundance was observed in all control plots. At Point Cook the apparent recovery of fished areas was sustained but at Flinders the recovery slowed or possibly waned.

Changes in Distribution

At both sites and during all surveys the distribution of abalone was found to be significantly aggregated (standardized Morisita index of dispersion >0.5, therefore >95% confident of aggregated pattern). Changes in the non-standardized Morisita index of dispersion indicated site-specific differences in distribution (Fig. 4). The Flinders plots showed a consistently higher degree of aggregation. This was indicative of there being many areas with no abalone and a few areas with many. The lower degree of aggregation at Point Cook was indicative of a more homogenous spatial distribution. These initial distributions influenced the change in distribution observed after fishing. At Flinders the removal of abalone from aggregations made the size of aggregations less extreme and lowered the degree of aggregation. At Point Cook the even removal of abalone across the entire area created grid cells with no abalone, thereby causing an increase in the degree of aggregation. The longer-term recovery after fishing generally showed a return toward the initial distribution.

The complete enumeration of all abalone within the plots meant that a Chi-square test of goodness-of-fit (Campbell & Clarke 1971) could be used to compare the observed and expected distributions of first, second, and third nearest-neighbor distances. These tests indicated that significantly aggregated patterns existed in all plots at both sites during each survey ($P < 0.01$, df = 5). Prior to fishing the distribution of first, second, and third nearest-neighbor distances was similar in fished and control plots at each site (Fig. 5 and Fig. 6). At both sites approximately 80% of abalone were located within 30 cm of their nearest neighbor. This aggregated pattern was more extreme at Flinders where about 50% of abalone were located within 5 cm of their nearest neighbor (compared with about 40% at Point Cook). Distances to second and third nearest neighbors were also less at Flinders than at Point Cook. About 25% of second and 10% of third nearest neighbors were located within 5 cm of the source abalone at Flinders compared with 10% and <5% respectively, at Point Cook. This pattern prior to fishing is indicative of the more aggregated pattern at Flinders and the more homogenous distribution at Point Cook.

The apparent impact of fishing was most noticeable at Flinders (Fig. 6). In both control and fished plots the proportion of first, second, and third nearest neighbors found close to the source abalone decreased during the first post fishing survey and the proportion located some distance from the source abalone had increased. This change was most noticeable in the fished plots. At Point Cook these changes were only noticeable in the fished plots (Fig. 5) and to a lesser extent than at Flinders. Changes in the distribution of

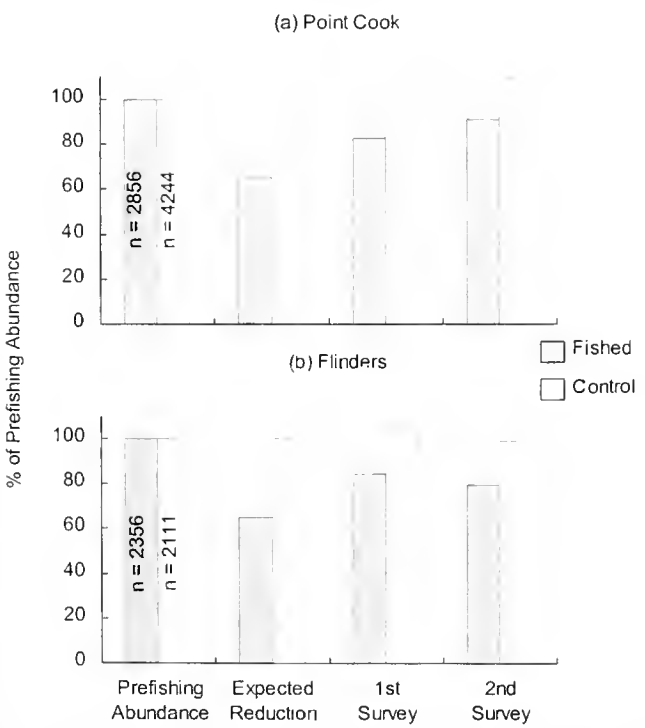

Figure 3. Changes in abundance of abalone within fished and control plots at (a) Point Cook and (b) Flinders over the course of the experiment.

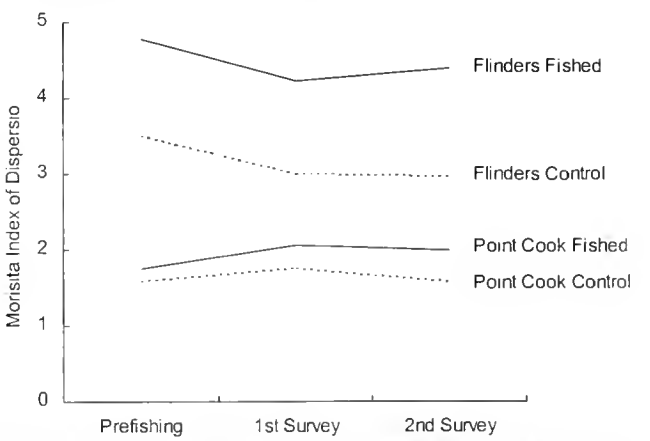

Figure 4. Changes in the non-standardized Morisita Index of Dispersion for abalone within fished and control plots at Point Cook and Flinders over the course of the experiment.

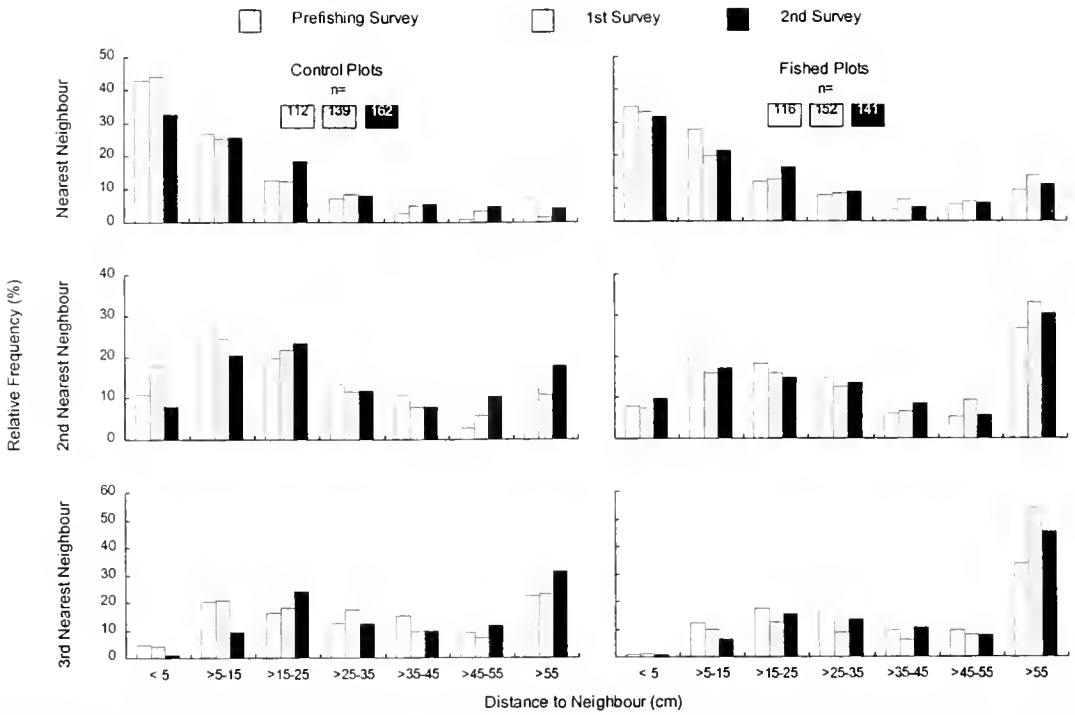

Figure 5. Relative frequency distributions of distances from abalone to their nearest, second nearest, and third nearest neighbors at Point Cook prior to fishing, and at each post-fishing survey.

nearest-neighbor distances in the control plots at Point Cook between the pre-fishing and first post-fishing survey were erratic. Between the first and second post-fishing surveys changes in these distributions in control plots at Point Cook remained erratic.

Conversely, by the second post-fishing survey the distribution of first, second, and third nearest neighbors at Flinders and in the fished plots at Point Cook had shown a general recovery toward the distribution found prior to fishing. At Flinders this recovery was most complete in the control plots. In fished plots the proportion of first, second, and third nearest neighboring abalone found

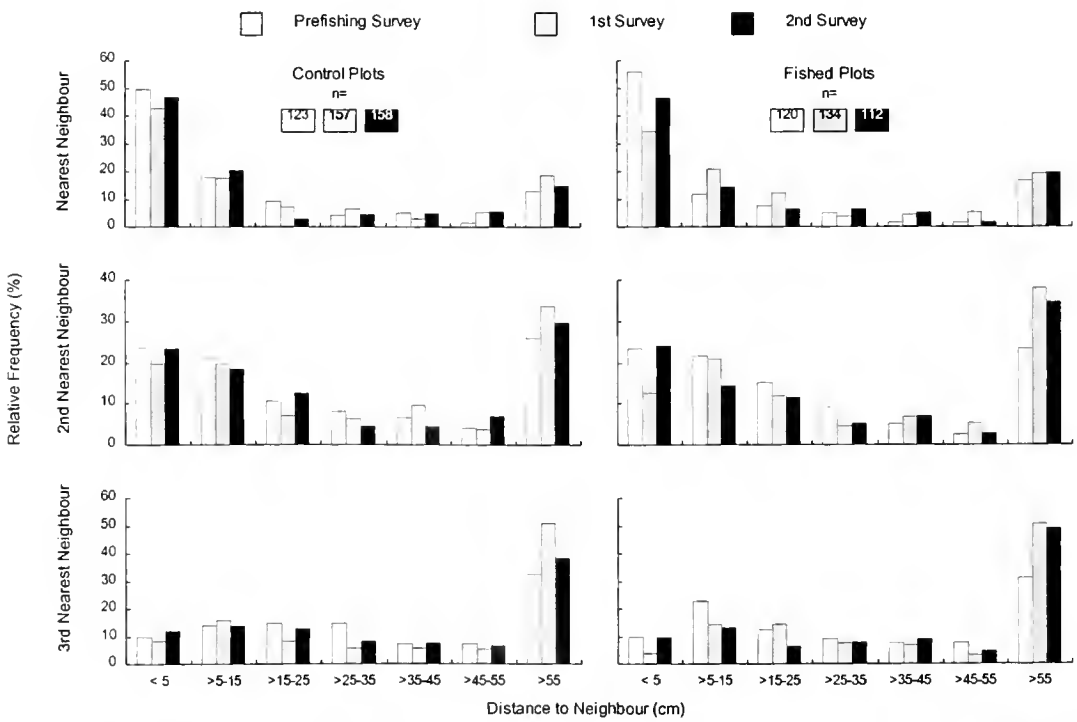

Figure 6. Relative frequency distributions of distances from abalone to their nearest, second nearest, and third nearest neighbors at Flinders prior to fishing, and at each post-fishing survey.

within 5 cm of the source abalone was similar on the second post-fishing survey to that found prior to fishing. In contrast, the increase noted on the first post-fishing survey in the proportion of abalone located some distance from the source abalone decreased only marginally on the second survey.

Emergence

Categorizing the shell cover of abalone proved to be difficult and imprecise. Therefore, these data were not used for analysis. Figure 7 shows the relative length frequency distributions of the abalone in fished areas at each site. Length frequency distributions before and after fishing did not show great change in the proportions of animals above and below the size limit.

Immigration

Figure 8 shows changes in the number of abalone counted on fixed transects around fished and control areas at each of the sites. At Point Cook there was a general and sustained decline in the number of abalone around fished areas after fishing. This result was not as apparent around control plots. At Flinders there was a decline in abundance around both fished and control areas but this decline was not as marked as at Point Cook.

The immigration of abalone tagged outside the plots is illustrated for the Point Cook site in Figure 9. The movement of abalone into control plots was just as significant as the movement into fished areas. While the number of tag movements at Flinders was not as great as at Point Cook, the results were similar.

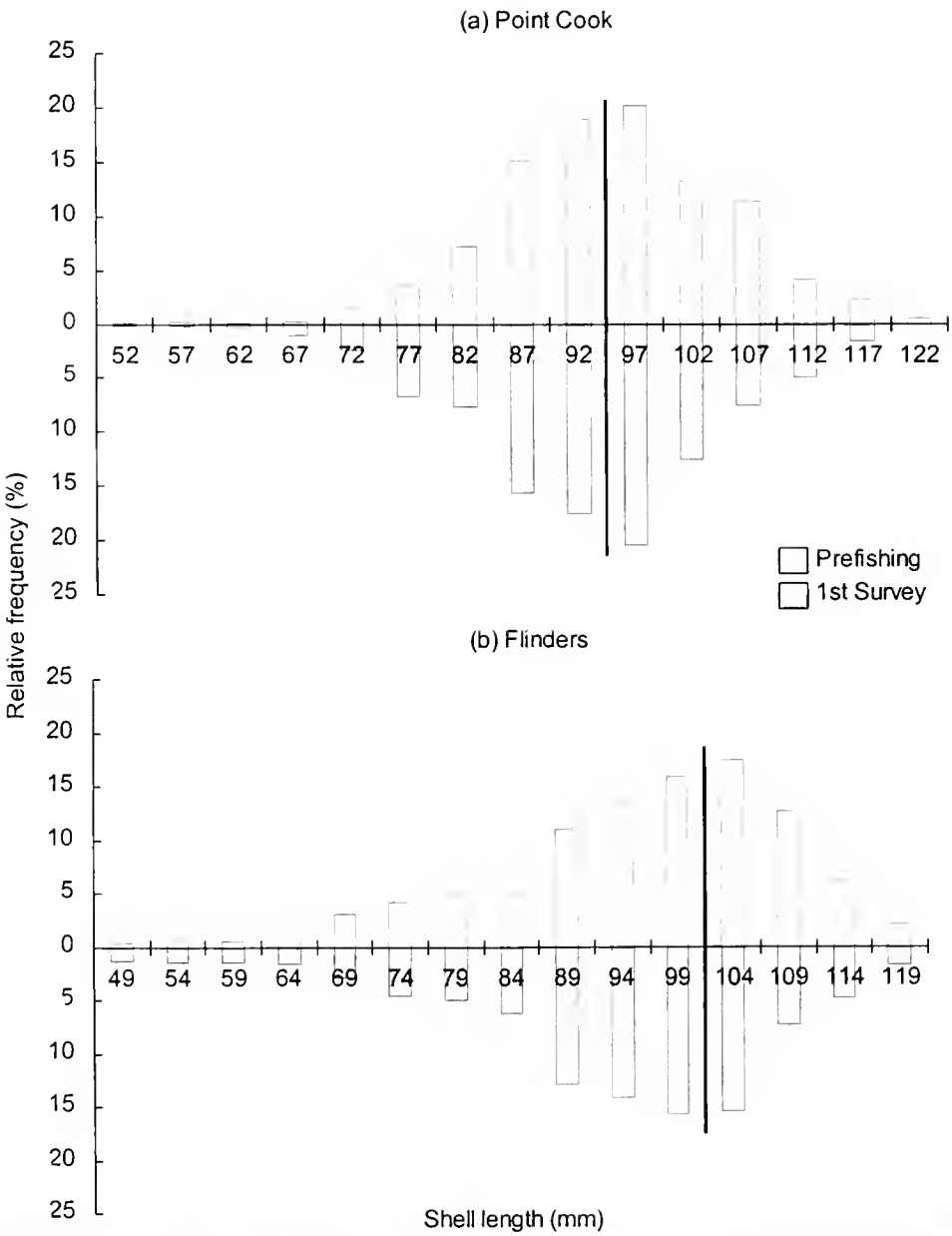

Figure 7. Relative length frequency distributions of abalone within fished plots at (a) Point Cook and (b) Flinders. The distribution prior to fishing is shown above the x-axis and the distribution after fishing is shown below the x-axis. Vertical lines indicate the size limits used at each site.

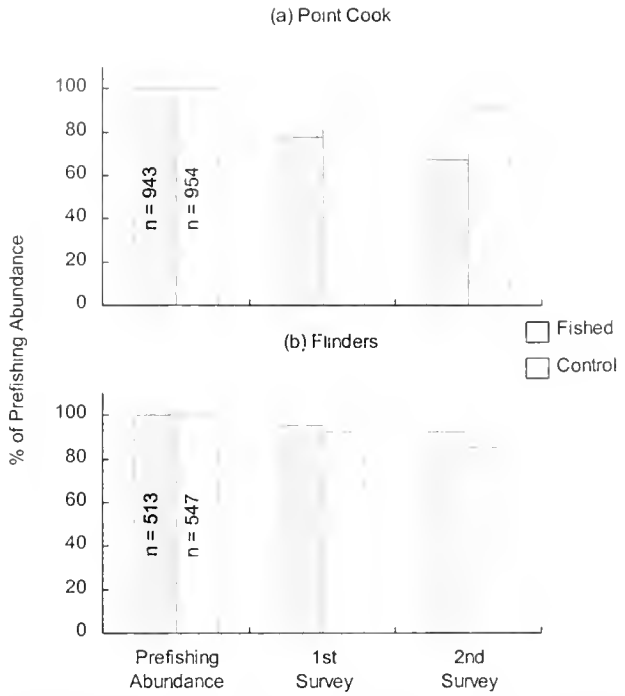

Figure 8. Changes in the abundance of abalone counted on fixed transects outside fished and control plots at (a) Point Cook and (b) Flinders over the course of the experiment.

Relocation

Figure 10 shows the number of abalone that moved and subsequently settled at each site plotted against the initial abundance of the cell in which they were tagged. At both sites abalone settled in cells of lower initial abundance than that of the cells in which they were tagged. This movement occurred in both control and fished plots but was more apparent in fished plots.

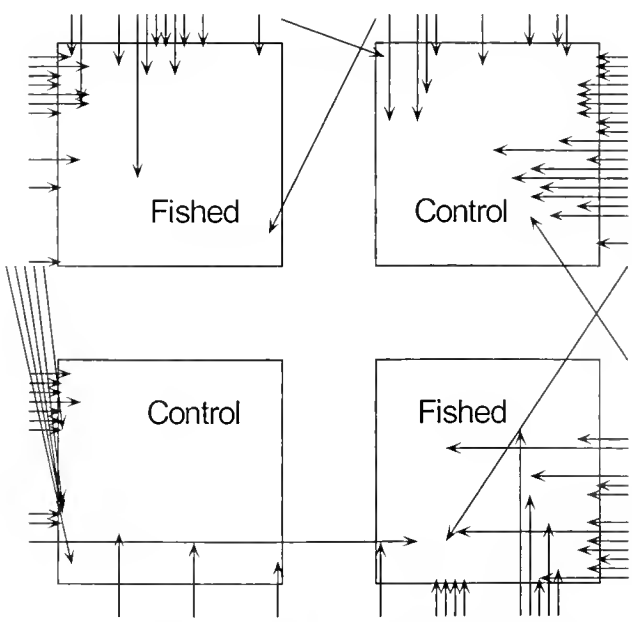

Figure 9. Schematic diagram showing the minimum distance moved of peripherally tagged abalone that moved into the plots at Point Cook.

DISCUSSION

Many traditional methods of fisheries stock assessment assume that movement-driven changes in spatial distribution have little impact on estimates of abundance or important parameters such as catchability and natural mortality. Such methods have been applied in abalone population surveys despite many anecdotes describing the propensity for abalone to re-aggregate after fishing and during spawning (Shepherd 1986b, McShane 1996, Hart & Gorfine 1997). Although there have been previous studies of abalone movement (Newman 1966, Poore 1972, Shepherd 1986a, Ault & DeMartini 1987, Prince 1992) and the frequency of aggregations (Shepherd 1986b, McShane 1996), a synthesis of these results has remained problematic. This study attempted to redress this deficiency by employing a combination of *in situ* tagging with fine-scale mapping of two distinctly different abalone populations. Controlled fishing of each population was conducted to test the hypothesis that removal of abalone by fishing stimulates movements that result in re-aggregation.

Experimental fishing clearly had an impact on the populations, evident as declines in the abundance of abalone counted in all fished areas. However, these reductions were less than those expected. Without fishing induced movement a 35% reduction in fished areas was expected and no change was expected in control areas. The increase in abundance in control areas, while not as great, may suggest a response to fishing at some distance. The fact that these changes in abundance within the plots were the opposite of those shown outside the plots suggested that recovery of the populations after fishing was occurring.

Substantial movement was observed and both populations showed a trend to return to their original spatial distributions. However, understanding how this redistribution was achieved proved difficult because contradictory results were obtained from two parts of the study. Analysis of the movements of tagged abalone showed an apparent dis-aggregation during the recovery, whereas the analysis of changes in the spatial distribution of abalone during the recovery suggested a re-aggregation. It was anticipated that the movement of tagged abalone would provide data that would best describe the nature of recovery of the abalone populations to fishing. Unfortunately these data were apparently the least reliable as the number of tagged abalone available for this analysis was only a small proportion of the tagged population. Furthermore, the repeated disturbance during the tagging, fishing, and re-surveying could well have caused abalone to disperse, reducing the likelihood of recapture, and resulting in dis-aggregation. Increased movement activity has been noted in other abalone species subject to disturbance (Shepherd & Godoy 1989, Werner et al. 1995). One of the important, but unforeseen, outcomes from this study was the impact that tagging-induced dispersal may have on estimates of natural mortality (Dixon et al. 1998).

Changes in the distribution of nearest-neighbor distances appeared to better describe the recovery of the populations after fishing. These data represented the entire population under study and were therefore less likely to be affected by tagging induced disturbances. These data suggested that restocking of aggregations occurred at both sites but that it was less noticeable at Point Cook where the abalone displayed a more homogenous distribution. The attenuated recovery at Flinders suggests that reformed aggregations contained fewer individuals than they did prior to fishing and that sparsely populated areas surrounding aggregations were not

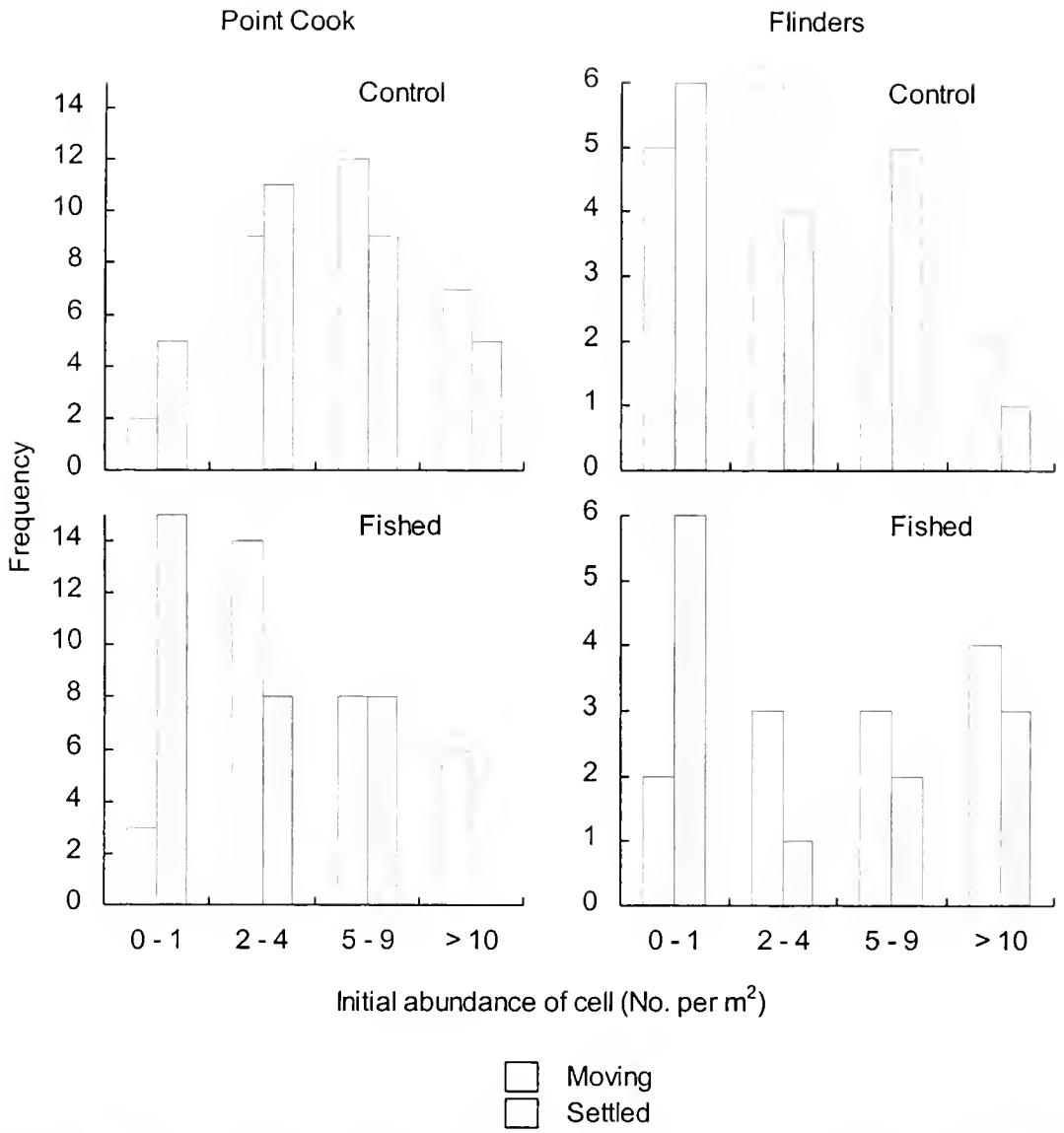

Figure 10. Position of moving and settled abalone relative to the initial abundance of the cell in which they were sighted.

quickly restocked following fishing. The recovery of a population through re-aggregation without complete restoration of pre-fishing population densities has important implications for area-based population surveys because such a re-distribution of abalone will mask depletion caused by fishing. Moreover, the tendency for recruited abalone to re-aggregate ensures that catchability will be maintained despite stock depletion. This means that abalone populations are more vulnerable to over-exploitation than populations of species whose catchability declines as stock numbers decrease. This is not surprising given the global history of abalone stock collapses.

Smaller abalone were not the prime source of re-aggregation particularly where emergence from crevices was a factor. This result is contrary to the conclusions of other studies (McShane & Smith 1989, Hart & Gorfine 1997, Hart et al. 1997). It is, however, consistent with the notion that smaller abalone seek the shelter afforded to them by cryptic habitat until they are large enough that the risks of occupying more exposed locations are outweighed by the benefits of improved food availability and the need to reproduce. Most of the observed movements involved relatively small displacements that, when considered together with post-fishing changes in abundance, suggest re-aggregation resulted from a series of contiguous displacements similar to a "domino effect." This implies that post-fishing spatial dynamics involves size-related competition for preferred home-sites.

Our results cast serious doubt on the validity of change-in-ratio methods that have sometimes been used for estimating abalone abundance (Nash et al. 1994). Our results indicate that violation of the critical assumption of equal catchability of two animal types (pre-recruits and recruits) is inevitable. Fine-scale hyper-stability in catch per unit effort (CPUE) is another consequence of re-aggregation and renders CPUE useless as an index of abundance except under conditions of severe depletion. This is not to suggest that instantaneous catch rate may not be sensitive to localized stock depletion. Immigration of abalone into fished areas, such as that suggested in this study, would further undermine the assumptions of change-in-ratio analyses. This will be even more apparent if immigration is most noticeable in recruits only.

Blacklip abalone populations can be regarded as comprised of

two parts, the aggregations that collectively constitute the stock commercial abalone divers are adept at targeting, and the more sparsely distributed abalone that serve to re-supply and thus maintain the aggregations after fishing. Although estimates of the numbers and sizes of aggregations provides a picture of the size of the stock, it is likely that these estimates tend to be hyper-stable whereas the density of sparsely distributed abalone may be a more sensitive indicator of the impact of fishing. Future research should be directed toward developing methods of abundance estimation that accommodate re-aggregation. Further analysis of our data using techniques developed for terrestrial ecosystems that estimate density using distances between nearest neighbors and distances from randomly selected sampling points offer promise in this regard (Byth & Ripley 1980, Byth 1982, Officer et al. 2000). Here, the distances are considered as radii that define areas occupied by both sparse and clumped individuals. As the degree of aggregation increases, the distances between nearest neighbors decreases while the distances from randomly selected points tends to increase. The geometric mean between the overall density of nearest neighbors and the overall density relative to random points gives an estimate of density with less bias than more conventional estimates from quadrats and transects. Regardless of the survey methodology employed, the results of this study indicate that aggregating behavior must be considered when assessing the impact of harvesting on blacklip abalone populations.

ACKNOWLEDGMENTS

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DISTANCE-BASED ABUNDANCE ESTIMATION FOR ABALONE

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ABSTRACT Indices of abundance are an important component of stock assessment models. Unfortunately, conventional attempts to estimate the abundance of abalone are hampered by the patchy spatial distribution characteristic of abalone. An alternative distance-based abundance estimator was evaluated by the simulated sampling of a large natural population of abalone whose relative positions had been accurately mapped. Distance-based abundance estimators can better accommodate the aggregated distribution of abalone. The simulated sampling of the real population of blacklip abalone (*Haliotis rubra* Leach) was used to examine the accuracy, bias, and sensitivity to changes in abundance of the distance-based approach. In each sample the distances from a randomly selected point and from a randomly selected abalone to the nearest abalone were used as the basis for a compound estimate of abundance. A Monte Carlo procedure was used to obtain percentile confidence limits about abundance estimates. The distance-based method was found to approximate the true abundance of the population and therefore may be useful as an indicator of absolute abundance. The method was also sensitive to changes in abundance. This sensitivity was examined by simulating the effects of fishing at varying rates of exploitation. Simulated reductions at moderate levels of exploitation (20–25% overall reduction) showed that the method was able to detect changes in abundance with reasonable confidence (90%). The resolution of some technical difficulties may enhance the ability of the distance-based method to detect changes in abundance in a real fishery.

KEY WORDS: *Haliotis rubra*, abalone, blacklip, stock assessment, abundance estimation, nearest neighbor distance, distance

INTRODUCTION

Blacklip abalone, *Haliotis rubra*, form the basis of a large and valuable fishery in southern Australian waters. Despite the economic importance of the fishery, stock assessment of blacklip abalone has remained problematic. Proper estimation of the sustainable yield from the fishery requires assessment of how the size of the stock changes over time. For assessment methods to be useful in routine monitoring they must: provide a reliable relative or absolute index of abundance for the stock, be sensitive to changes in abundance in the population, and be easily applied across a representative proportion of the fishery.

Fishery-dependent methods of stock assessment often fail for abalone fisheries because of the inherent hyper-stability in catch-rate indices (Breen 1992, McShane 1994). The tendency of blacklip abalone to live in aggregations renders them more vulnerable to overfishing by increasing their catchability. Divers are able to maintain high catch rates by moving from aggregation to aggregation and by relocating to a different area once catch rates drop below an acceptable level. If the abalone remaining after fishing then re-aggregate, a diver returning to the area may well fish the area at a similar catch rate to the first excursion. These processes may all contribute to hyper-stability in catch rates and conceal real fluctuations in population size due to local depletion.

Unfortunately, fishery-independent methods of assessment are also difficult to apply to abalone populations. Area-based abundance estimators can provide accurate measurement of abundance and an ability to detect change (Hart & Gorfine 1997, Hart et al. 1997). However, the patchy distribution of abalone unfortunately introduces high variance into such estimates and sample sizes must therefore be extremely high to achieve acceptable levels of precision (Nash 1995). Methods such as mark-recapture and change-in-ratio both may show reasonable precision and sensitivity (Hart and Gorfine 1997). Unfortunately, the sampling intensity needed to achieve this makes these measures useful only for the assessment of small populations. The time required to apply such measures across a large fishery would make them prohibitively expensive as a routine monitoring tool.

Fishery-independent assessment methods that are less time consuming offer scope for the monitoring of large areas but may forsake precision. Estimates provided by techniques such as timed collections suffer from variation in collection efficiency between divers (Shepherd 1985, McShane 1994) and in sea conditions at the time of sampling. Such methods are also prone to hyper-stability due to the tendency of abalone to aggregate and the unintentional attraction of research divers to these aggregations (McShane 1994). Where the research diver spends most time within an aggregation handling abalone, rather than searching, the estimated density tends unrealistically toward infinity (Hart et al. 1997).

The development of assessment methods that better handle the aggregated distribution of abalone would clearly be an advantage. Distance-based methods of abundance estimation offer scope for species such as abalone that are largely sedentary and aggregated. Techniques developed for terrestrial ecosystems that estimate density using distances between nearest neighbors and distances between randomly selected sampling points and the nearest individual of the species of interest offer potentially workable methods (Byth & Ripley 1980, Byth 1982). As the degree of aggregation increases, the distances between nearest neighbors tend to decrease while the distances from randomly selected points to individuals tend to increase. Compound estimates of the overall density using these two measures may therefore accommodate a wider range of spatial distribution types than more conventional abundance estimates (Diggle 1975). While such methods have been in existence for some time (Clark & Evans 1954) they have not been applied to abalone. This is perhaps because the data requirements necessary to test the efficacy of the methods are particularly difficult to achieve—samples are required for a population of known abundance and distribution.

The objective of this study was to evaluate the efficacy of a relatively simple distance-based abundance estimator. This was done using simulated measurements and removals of abalone from a blacklip abalone population of known abundance and spatial

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distribution. The evaluation examined the accuracy, precision, and sensitivity to changes in abundance of the estimator.

MATERIALS AND METHODS

Study Site

The mapping of abalone distributions was undertaken at Point Cook, Victoria, Australia (37°55.893′ South, 144°47.104′ East). The site was 3 to 4 m in depth and was not subject to strong swell or current. The reef consisted of basalt boulders, rarely over 1 m in height, on a sandy substrate. There was very little cryptic habitat that could not be thoroughly searched for abalone.

The study site covered an area of 58 × 58 m, subdivided into four plots of 24 × 24 m separated by corridors 10 m wide. Each plot was further divided into 1 m^2 cells using a grid system. The grid was used to record the position of all abalone observed within the plot within an accuracy of ±10 cm. To simplify the application of the computer simulation procedure the 10-m corridors between plots were removed, creating one plot measuring 48 × 48 m.

Simulation Procedure

The position of all abalone observed by divers in the plot was recorded in two dimensions as an X-Y coordinate and saved in a Microsoft Excel spreadsheet (Fig. 1). A total of 8009 abalone was observed by divers in the plot. The length composition of the population was determined for a sample of 1809 abalone measured in the study area. To allow for comparisons between the size structure of abalone occupying densely populated habitat and those in sparsely populated areas, lengths had been recorded separately for abalone in each type of habitat. Each of the 8009 abalone was assigned a length from the known length composition data.

An interactive macro program was written in Visual Basic to calculate a series of distances between source positions (either randomly chosen points or randomly chosen abalone) and the nearest abalone. The method assumes that distances can be measured from source positions within the plot to abalone located outside the plot. Because there was no data for abalone outside the plot, source positions were restricted to lie inside a 2-m border region around the plot (Fig. 1). It was possible to measure from a source position located within the inner plot to an abalone located within the border region but no measurements originated within the border. In all simulations reported here the border region used was 2 m. This reduced the size of the area from which source positions could be chosen to 44 × 44 m. 6596 abalone were observed within the 44 × 44 m inner plot.

Calculation of Randomly Chosen Point to Nearest Abalone Distances

A random number generator was used to generate the required number of randomly chosen points within the inner plot. For every randomly chosen point the distance to the nearest abalone was recorded. The nearest abalone could be located in the 2-m border region outside the inner plot.

Calculation of Randomly Chosen Abalone to Nearest Abalone Distances

Because the number of abalone within the plot was known it was possible to assign each abalone a random number. A table of random numbers was then used to choose abalone located within the inner plot from which distances to the nearest abalone would be calculated. The nearest abalone could be located in the 2-m border region outside the inner plot. The number of randomly chosen abalone from which to calculate distances could be specified. The macro prevented abalone from being chosen more than once in any sample taken from the plot. This avoided the remeasurement of nearest neighbor distances from the same abalone.

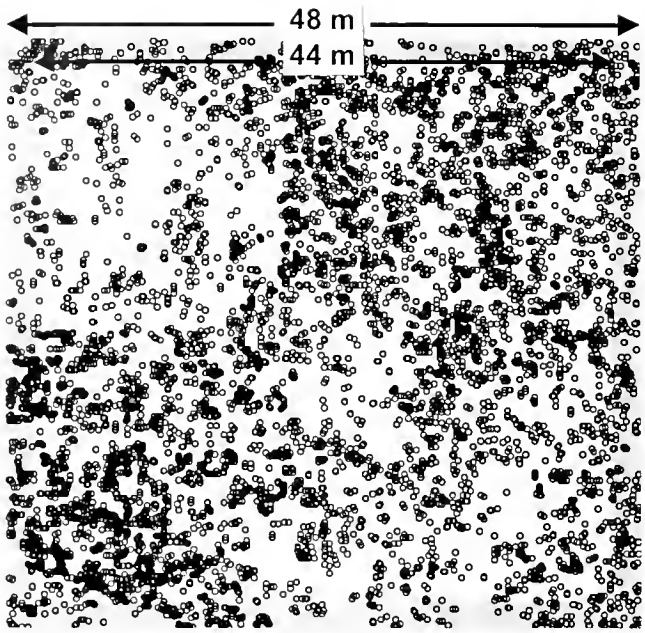

Figure 1. Diagram showing the position of all 8009 abalone within the study area. Abalone are plotted as unfilled circles several times their actual size. Where abalone are densely aggregated the plotted circles overlap creating dark patches. Source positions for either randomly chosen points or randomly chosen abalone were prevented from originating within a 2-m border region (shaded area) to prevent the possibility that the nearest abalone to a source position was outside the mapped region. However, it was possible to measure from abalone located within the inner plot to abalone located within the border region.

For every randomly chosen abalone the distance to the nearest abalone was calculated. The lengths assigned to each abalone were used to compare the calculated nearest neighbor distance with the minimum possible distance between the centers of each abalone. The greater of these two distances was recorded. This was done to avoid assigning nearest neighbor distances smaller than if nearest neighbors were touching.

Calculation of Distance-based Density Estimates

The formula suggested by Byth and Ripley (1980) was used to generate estimates of population density (Eq. 1):

$$\hat{N} = \frac{n}{\pi \sum (r_i^2)}$$

Where

$\hat{N}$ is an estimate of population density,

n is the sample size (number of distances measured), and

r_i is the distance from the ith random source position to the nearest abalone.

Density estimates were calculated separately from the series of randomly chosen point-abalone distances and from the series of randomly chosen abalone-abalone distances. Unfortunately the density estimates derived from each type of calculation are sensitive to the spatial pattern of the organisms under study (Diggle 1975, Krebs 1989). Diggle (1975) found that as the degree of

population aggregation increased, point-organism distances also tended to increase resulting in underestimates of population density. Conversely, organism-organism distances tended to decrease and result in overestimation of population density. He showed that an unbiased estimate of the population density could be obtained by calculating the geometric mean of the point-organism and organism-organism density estimates. In this study Diggle's protocol was followed and a compound and theoretically unbiased estimate of density was calculated as (Eq. 2):

$$\hat{N} = \sqrt{\hat{N}_{RCPt}\,\hat{N}_{RCAb}}$$

Where

$\hat{N}$ is a compound estimate of population density, and

$\hat{N}_{RCPt}$ and $\hat{N}_{RCAb}$ are respectively the randomly chosen point-abalone and randomly chosen abalone-abalone estimates of population density (derived from Equation 1).

Calculation of Expected Densities

The expected density of abalone in the plot was calculated as the total number of abalone within the inner plot divided by the total area of the entire plot (48 × 48 m = 2304 m^2). To more closely reflect the approach taken by the distance-based density estimation, the number of abalone within the inner plot was divided by the total area, rather than the area of the inner plot. The distance-based method estimates density for a larger area than that of the inner plot because it was possible to measure from a source position located within the inner plot to an abalone located within the 2-m border region.

Expected densities were calculated for the initial population and for three levels of population reduction. The levels of reduction simulated were 25%, 50%, and 75% less than the initial population size. Little difference was found between the size composition of abalone located in densely populated habitat with that of abalone located in sparsely populated areas. The simulated removal of abalone from the plot was therefore done randomly to effect each level of population reduction.

Estimation of Optimal Sample Size

By successively increasing the number of distance measurements used in the calculation of the compound distance-based estimate, the simulator was used to determine the sampling intensity required for consistent density estimation. The number of distance measurements of each type was increased from 50 to 800 in steps of 50. Five iterations were run at each step to allow variability to be examined for each number of measurements.

Estimation of the Minimum Detectable Difference

Subsequent distance-based estimates of the initial population density were calculated from 250 distance measurements of each type of density estimate. This number was chosen for two reasons: Further increases in the number of distance measurements did not appear to greatly increase the precision of density estimates, and, 250 was thought to be a practical number of measurements to take if conducting the sampling in the field.

250 randomly chosen abalone represented 3.8% of the initial population of 6596 abalone within the inner plot of the study area. 100 iterations of the simulation procedure were run to generate a distribution of estimated densities for the population within the entire plot. 80%, 90%, and 95% percentile confidence limits were calculated from this distribution.

To estimate the minimum detectable difference the population was reduced in successive steps and the simulation procedure rerun 100 times at each level of reduction. The number of distance measurements of each type was kept at the same percentage (3.8%) of the residual population within the inner plot to maintain the same level of sampling intensity as that used in the estimation of density of the initial population. 80%, 90%, and 95% percentile confidence limits were calculated for each level of reduction.

A comparison was made between the 100 estimated densities calculated at each level of reduction and the corresponding expected densities. The relationship between the two was described using linear regression. Regression lines were also drawn between the various upper and lower percentile confidence limits. These relationships were used to estimate the ability of the method to detect changes in abundance at various levels of confidence and population density.

RESULTS

Estimation of Optimal Sample Size

Successive increases in the number of distance measurements used in each simulation improved the consistency of density estimates calculated for the initial population (Fig. 2). Variability in repeated simulations reduced markedly when 300 or more distances of each type were measured. Density estimates of the initial population approached a central limit of about 2.7 abalone/m^2.

Estimation of the Minimum Detectable Difference

Negligible differences in size structure were found between abalone in densely populated habitat and those in sparsely populated areas (Fig. 3). This justified the random removal of abalone from the plot to affect each level of population reduction.

Estimates of density were less variable at reduced population sizes (Table 1, Fig. 4) despite there being no increase in sampling intensity in simulations run at lower population sizes. Density

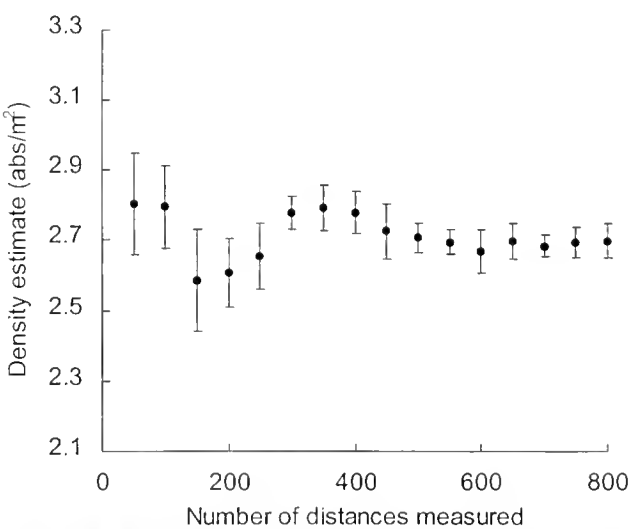

Figure 2. Average density estimates (±SE) calculated by the simulator at various sampling intensities. Five iterations were run at each sampling intensity. The sampling intensity is indicated by the number of distances measured of each type (i.e., 200 indicates that 200 randomly chosen point-abalone and 200 randomly chosen abalone-abalone distances were measured).

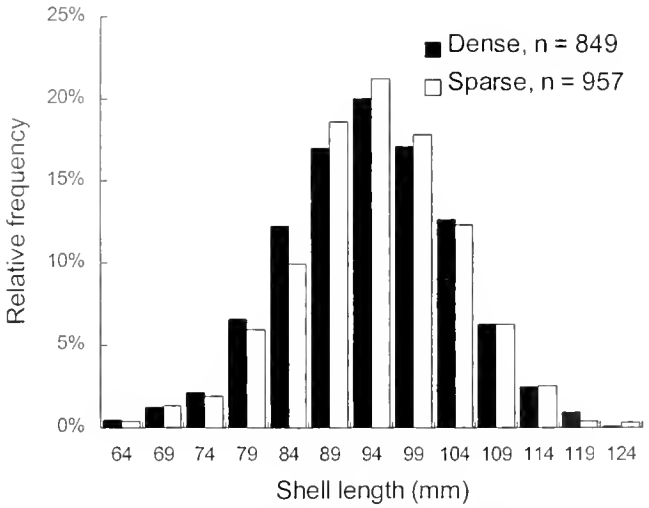

Figure 3. Comparison of the size structure of abalone collected from densely populated habitat and sparsely populated habitat at the Point Cook site. There is little difference in the size structure of abalone collected from each habitat type and above the minimum legal length of 100 mm these differences are negligible.

estimates at each population size were normally distributed and reflected relative reductions in population size (Fig. 4).

The relationships between estimated and expected densities, and between the various upper and lower percentile confidence limits were linear (Fig. 5). Estimated densities closely estimated the true density of the population, and estimated reductions were close to the true levels of reduction (Table 1). Estimated densities appeared to be a good indicator of absolute abundance. This was demonstrated by the strong proportional relationship found between estimated and absolute abundance (Estimated density = 0.92 × Expected density + 0.10, $R^2 = 0.96$). The ability to detect proportional differences in abundance was dependent on the size of the population and declined in proportional terms with reducing population size.

The linear relationship between estimated and expected densities (and the confidence limits about this relationship) allowed an evaluation of the power of the method to detect changes in abundance at a range of initial population densities. At greater initial population densities (i.e., >3 abalone/m^2) the method was able to detect declines in abundance of as little as 22% with 90% confidence (Fig. 6A). Power to detect declines with greater levels of confidence was high at greater initial population densities (e.g., at an initial density estimate of 3 abalone/m^2, declines in abundance of 24% could be detected with 95% confidence) (Fig. 6A). Power to detect proportional declines in abundance reduced markedly with declining initial density estimates less than 1 abalone/m^2 (Fig. 6A). Power to detect increases in abundance followed a similar pattern to that for detection of declines (Fig. 6B). At an initial population density of 3 abalone/m^2 the method was able to detect increases in abundance of about 26% with 90% confidence (Fig. 6B).

DISCUSSION

The distance-based abundance estimator described here was able to detect measurable changes in the absolute abundance of an abalone population. The minimum differences detectable were relatively small even at reasonably high levels of confidence. The method appears to satisfy two primary requirements of abundance estimation methods: it is a good indicator of abundance, and it is able to detect changes in abundance. A third requirement, that the method be easily applied across the fishery, was not examined completely in this study. The technical requirements of the method appear to be minor. The time consuming repeated surveys characteristic of change-in-ratio and mark-recapture methods are not required. The method therefore minimizes reliance on prolonged periods of good diving conditions. The measurements required are relatively simple to achieve and the skill required to take the measurements would probably be acquired quickly by an observer with limited experience. This satisfies the need of long-term monitoring programs to accommodate changes in the survey team and avoids the requirement for a standard dive team when using timed collection methods.

The simulated sampling using real field data presented here suggests that the method has the accuracy of area-based measures but the ease of application of timed collections. The levels of sampling demonstrated here do not represent an insurmountable amount of work. At lower densities the number of measurements that need to be taken could be increased. The method accommodates an uneven amount of randomly chosen point-abalone and randomly chosen abalone-abalone measurements, making it adaptable to field situations. Furthermore, by considering the randomly chosen point-abalone and randomly chosen abalone-abalone density estimates separately, the method provides an opportunity to describe the patchiness and patch density in the population and

TABLE 1.

Results of simulations performed at various population sizes.

Population Size	Number of Distances Measured	Population Reduction		Density (abs/m^2)		Percentiles	
		Actual	Estimated	Actual	Estimated	10th	90th
6596	250	–	–	2.86	$2.73_{0.19}$	2.40	3.06
4943	187	25%	25%	2.15	$2.05_{0.16}$	1.81	2.32
3300	124	50%	48%	1.43	$1.42_{0.15}$	1.16	1.66
1648	62	75%	72%	0.72	$0.75_{0.09}$	0.63	0.90

Population reductions were effected over the entire plot. Therefore the population size of reduced populations represents the average number of abalone remaining within the inner plot. To maintain the same relative sampling intensity the number of distances measured was less for simulations performed on reduced populations. The number of distances measured indicates the number measured of each type (i.e., 62 indicates that 62 randomly chosen point-abalone and 62 randomly chosen abalone-abalone distances were measured). Estimated reductions were calculated by comparing the estimated density of reduced population sizes with that of the initial population. Actual densities were calculated as the population size divided by the area of the entire plot (2304 m^2). The estimated density is the mean of 100 density estimates calculated at each population size. Sub-scripted numbers indicate the standard deviation about the mean.

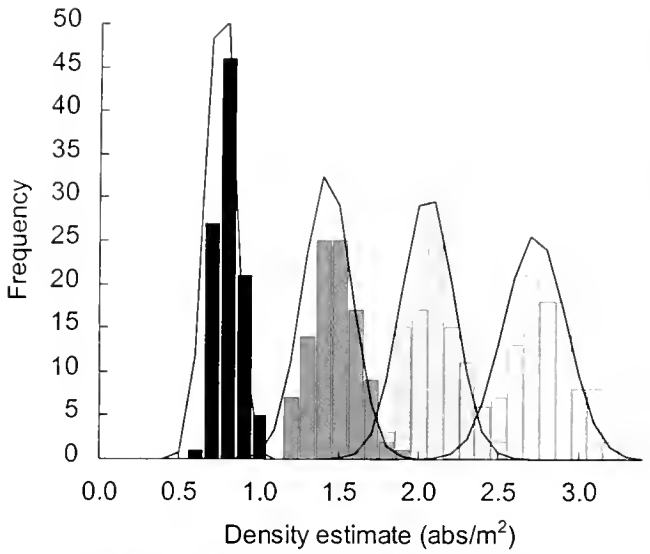

Figure 4. Distributions of density estimates generated from 100 simulations calculated at various population sizes: initial population (white bars), 25% reduction (light-shaded bars), 50% reduction (dark-shaded bars) and 75% reduction (black bars). Overlaid lines indicate normal distributions fitted to the estimated data for each population size. Note that the variance reduces as the mean density estimate decreases.

changes in these measures over time. Information on the patch structure of abalone populations may be an important indicator of productivity (McShane 1995, Taylor et al. 2000), but this information is not collected by most monitoring methods.

If the field requirements of the method could be satisfied then the method could be particularly useful as a routine estimator of abundance. However, the simulations reveal several limitations of the method and indicate some further areas for investigation. Perhaps the most important limitation of the analysis presented here is that the fishing patterns simulated were not real. The removal of abalone was effected by random selection of abalone from the entire plot. True fishing patterns are focused on aggregations of abalone (Hart et al. 1997). Random fishing assumes that all fish in the plot are of legal size and equally likely to be removed. In most abalone fisheries adherence to minimum size limits makes only a proportion of the population available to fishing. If the size structure of abalone occupying sparsely populated habitat differs from that in aggregations, fishing will dramatically affect the spatial structure of the population. In this study no evidence for differing size structure between densely and sparsely populated habitats was found in the Point Cook population. Similar results have been found at another site (Officer et al. 2000).

Even if no differences exist between size structures in densely and sparsely populated habitat, fishing patterns that focus on aggregations are likely to have both immediate and prolonged effects on the population. In the short term, the removal of abalone from aggregations would create a more dispersed and homogenous distribution pattern after fishing. However, there is evidence that abalone are capable of moving considerable distances if disturbed and that this enables re-aggregation into preferred home-sites to occur rapidly (Gorfine et al. 1998, Officer et al. 2000). At the Point Cook site this behavior returned the distribution of abalone to the initial distribution pattern (albeit at a lower density) within weeks of intensive fishing (Gorfine et al. 1998, Officer et al. 2000). The random removal of abalone simulated here is therefore likely to

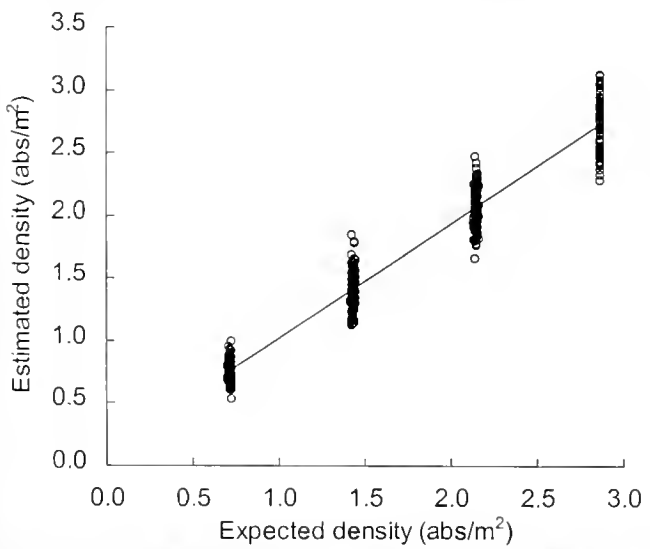

Figure 5. Relationship between estimated and expected density (Estimated density = 0.92 × Expected density + 0.10, $R^2 = 0.96$). Dotted lines indicate 90% confidence intervals.

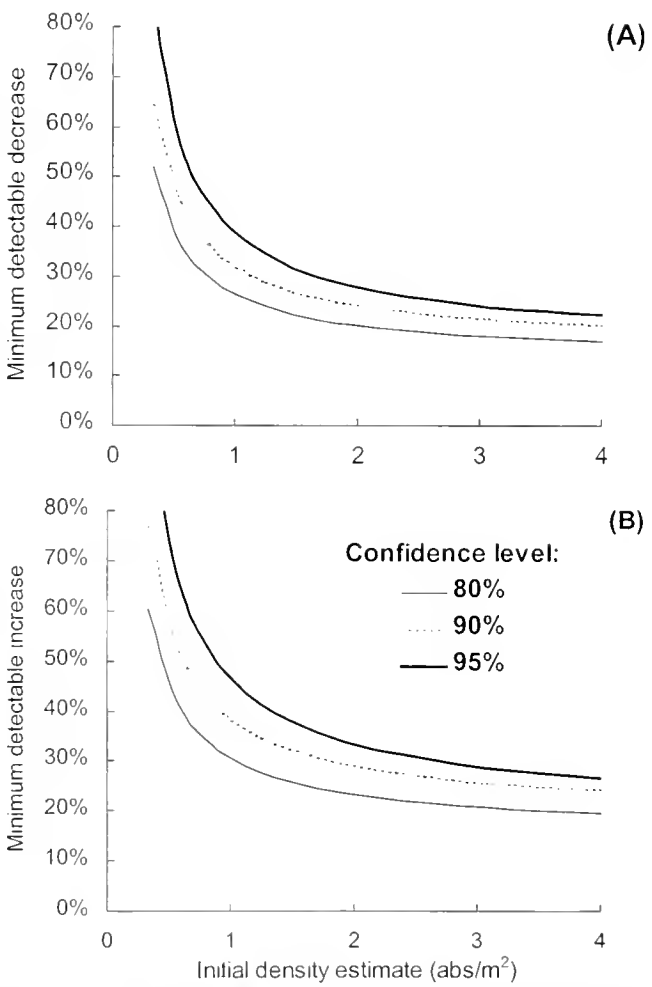

Figure 6. Power of the distance-based method to detect change. Minimum detectable changes are expressed as a percentage of the initial density estimate for (A) the minimum detectable proportion decrease, and (B) the minimum detectable proportion increase.

approximate the distribution of abalone found in the real Point Cook population several weeks after fishing.

Whether or not this relationship would hold generally is uncertain. In a study of the movement and re-aggregation of abalone, Officer et al. (2000) subjected the Point Cook site and a second abalone population (Flinders) to similar levels of experimental fishing. At Flinders the reef had much greater relief and cryptic habitat than at Point Cook and the abalone were less abundant but more patchily distributed. Recovery of the initial distribution pattern took much longer at Flinders (Officer et al. 2000). The Point Cook site was unusual in that it contained little cryptic habitat and low relief. This apparent success of the distance-based method at Point Cook may result from the two-dimensional area assumed by the estimator correlating well with the actual area of the site. Testing the efficacy of the distance-based method in areas of more complex topography and cryptic habitat will require further experimental work. Unfortunately the role of the cryptic component of abalone stocks remains poorly understood despite its importance to stock assessment (Haddon 2000).

Another shortcoming of the method is that it is only sensitive to changes in abundance when the population density is relatively high. In this respect the method differs from other assessment techniques that may become hyper-stable at higher population densities [e.g., the timed collection (Hart & Gorfine 1997, Hart et al. 1997)]. Fortunately, at lower population densities methods such as the timed collection come into their own as measures of relative abundance. A benefit of applying the method at higher population densities is that the area that has to be searched between nearest neighbors by research divers will tend to decrease. This will increase the efficiency of the method in the field. The method may therefore be particularly useful for *H. rubra* populations in Tasmania where the abundance of abalone appears to be relatively high and the amount of coast that needs to be monitored is extremely large.

There are also some technical limitations to the simulations applied here. Designing a field protocol that selects source positions and abalone randomly will not be a trivial task (Ripley 1981). Selecting a randomly chosen point underwater is relatively easy. The main problem may occur when the point falls on inappropriate habitat. This suggests a need to stratify sampling by habitat when applying the distance-based method. Selection of a randomly chosen abalone is far more complicated and may be a source of potential bias in the method (Ripley 1981). For the method to remain effective it will be important to avoid one of the shortcomings of the timed collection procedure: attraction of research divers to aggregations. The apparent utility of the method as a sensitive abundance estimator suggests that attempts to overcome these difficulties warrant serious attention.

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The data used in these simulations could not have been collected without the support of the Victorian abalone industry. Commercial abalone divers refrained from diving at the study location during the course of the study. Mapping the position of over 8000 abalone required substantial effort from the project's field staff. Assistance with vessel operations and diving support was provided within MAFRI by Mark Ferrier, David Beyer, Dale Thomson, and Ian Duckworth. Charter vessels were provided by Graham Leckie (Ultimate Fishing Charters) and Alistair MacDonald (Port Campbell SCUBA and Marine). Diving contractors employed on the project included David Forbes and Bruce Waters of Aquatic Research Support Services, Rod Knights from Professional Diving Services, Geoff Rodda from Peninsula Diving Instruction, and Michael Callan. The research divers collectively accumulated 1000 hours underwater during the surveys. We are extremely grateful to Joanne Merry who generously volunteered her time and expertise to support the field team with the logging of dives and the management of data collection while at sea, and who subsequently entered and processed the data on computer. The initial project was funded by the Fisheries Research and Development Corporation (Project 95/165) and the Marine and Freshwater Resources Institute.

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DIVER BEHAVIOUR AND ITS INFLUENCE ON ASSESSMENTS OF A QUOTA-MANAGED ABALONE FISHERY

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ABSTRACT During 1998, we initiated an on-board observer program to gain a better understanding of spatial and temporal patterns of catch and effort in the blacklip abalone fishery of Victoria, Australia. Although average catch per unit effort (CPUE) for this fishery has been increasing, there has also been significant spatial contraction of the fishing grounds away from reefs of low productivity as a consequence of quota introduction during 1988 to 1989. It is this shift away from reefs of low productivity rather than an increase in abalone abundance that is responsible for the trend in CPUE. From our on-board observations, divers do not operate in an area if they believe that they will not meet their daily catch expectations; they have a relatively high catch rate threshold for deciding when to shift to another reef. Catch rates per bag of abalone are several times higher than the daily reported CPUE rates, but vary substantially. We conclude that the incentive to maintain high catch rate thresholds for cessation of fishing has led to patterns of rotation of effort at the reef scale that tend to mitigate against high rates of exploitation. This has provided an effective tier of fine-scale self-governance below that controlled by management regulations. However, contemporary changes in the fishery, such as reductions in the number of divers who own access entitlements and their levels of experience, may lead to reduced catch rate thresholds and unfavorable patterns of fishing behavior. There is also a tendency for divers who have recently entered the fishery to concentrate their effort on shallower reefs for reasons of health and safety. Although there is no evidence to date that the over-all status of Victorian abalone stocks has been compromised by these changes, managers need to be aware that regulations affect diver behavior and that it is the divers not the resource that is managed. Our studies reinforce the importance of identifying and promoting behaviors among divers that are desirable in terms of sustainable production within the context of contemporary management strategies.

KEY WORDS: quotas, management, fishing behavior, abalone

INTRODUCTION

Although quota management is ubiquitous among productive abalone fisheries in the Southern Hemisphere, management agencies in the Northern Hemisphere are still considering the merits of quotas as output controls for abalone (CDFG 1997). In an assessment of the efficacy of quota management, it would be imprudent to assume that total allowable catches (TACs) per se have been the reason for the relatively prosperous state of abalone fisheries in the Southern Hemisphere. Quota management regimes are usually implemented over broad scales that provide considerable latitude for abalone divers to choose where and when to expend their fishing effort. For example, the abalone fishery in Victoria, Australia, which provides about 14% of global wild-stock production and spans about 1,500 km of coastline, is divided into three relatively large quota management zones that are not subject to seasonal closure (Fig. 1). Such expansive zones do not facilitate control of catch outputs at the metapopulation scale that delimits population recruitment (Shepherd & Baker 1998).

Not only may quota management regimes not be as effective in controlling outputs as intended, they may also have inadvertent effects on diver behavior. Prince and Shepherd (1992) identified potential for spatial maldistribution of effort arising from the implementation of quotas, with overfishing of the more productive areas of the fishing grounds proximal to port and underutilization of the more remote and less productive reefs. Since the introduction of TACs in the Victorian fishery, the fishing grounds have contracted substantially. There has been a progressive shift in effort away from reefs with lower catch rates toward more highly productive reefs (Gorfine et al. this publication). Although improbable, there are no regulatory constraints to prevent divers within a management zone from intensively harvesting the TAC from only one region in a relatively short time period (currently an aggregate of 50–55 days effort per diver is required to harvest annual quota allocations in Victoria). Quotas have also brought about structural changes in the Victorian abalone fishery. Mean experience levels among those participating in the catching sector has decreased markedly in all zones of the Victorian fishery since quotas were introduced. At the same time, the number of fishery access license owners choosing to engage the services of contract divers to harvest their quota allocations has increased commensurately (Fig. 2). It is reasonable to speculate that some impetus for this transition from owner-operator to contract diver came from increased profitability associated with an escalation in market demand for Australian abalone products that saw the beach price more than double between 1991 and 1992 and 1993 to 1994 (Fisheries Victoria 1998).

Despite the potential for intense fishing pressure to cause serial depletion of the more productive reefs, this has not been manifest in the Victorian abalone resource (McShane 1992), although recent modeling predicts a slow decline in relative biomass (Gorfine & Dixon 2000), and there are some recent instances of localized depletion. Possible explanations for the apparent sustainability of Victorian abalone stocks include:

1. Reductions in real and latent effort through a combination of limited entry, nontransferability, and diver attrition during the two decades preceding quota introduction in 1988 (Sanders & Beinssen 1972, McShane 1992). Further effort reductions when transferability on a "two divers out for each diver in" basis was introduced during 1984.
2. Divers varying their effort at a given reef considerably from year to year. In many instances, effort is rotated among reefs to maintain catch rates and allow substocks on recently fished reefs time to recover (McShane 1992). This is akin to the rotational harvest strategies discussed by Perry et al. (1999). However, although many reefs may recover periodically over short time scales, the extent to which this is sustainable for each reef complex is presently unclear.

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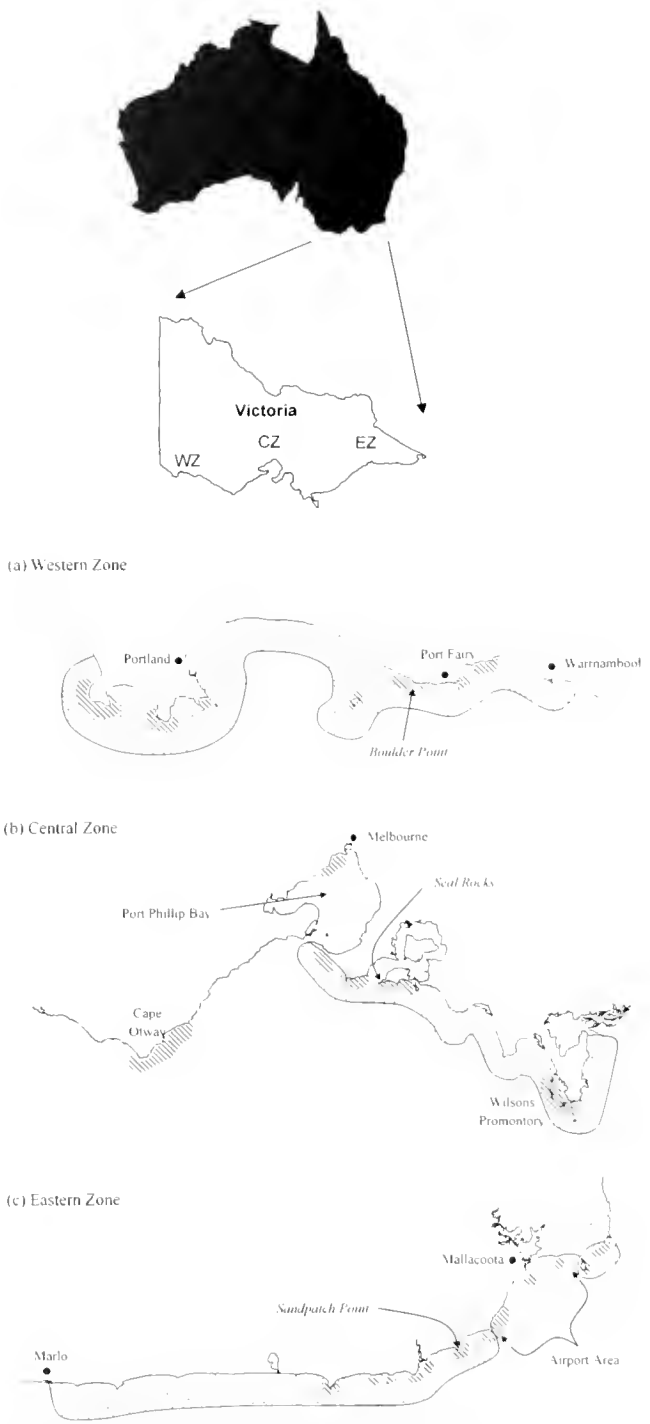

Figure 1. Fishing grounds (stippled) and key reef areas (hatched) in the (a) Western Zone, (b) Central Zone and (c) Eastern Zone, that were included in on-board observations of the Victorian abalone fishery.

3. Self-imposed daily quotas. For example, in the Eastern Zone of the Victorian fishery, the Abalone Fishermen's Cooperative, which includes about 21 out of the 23 fishery access licenses, has a self-imposed daily limit of twelve bins (approximately 480 kg live weight) per diver. Similar forms of self-governance have been evident among Victorian divers generally during the formative first decade of the fishery (Sanders & Beinssen 1972).

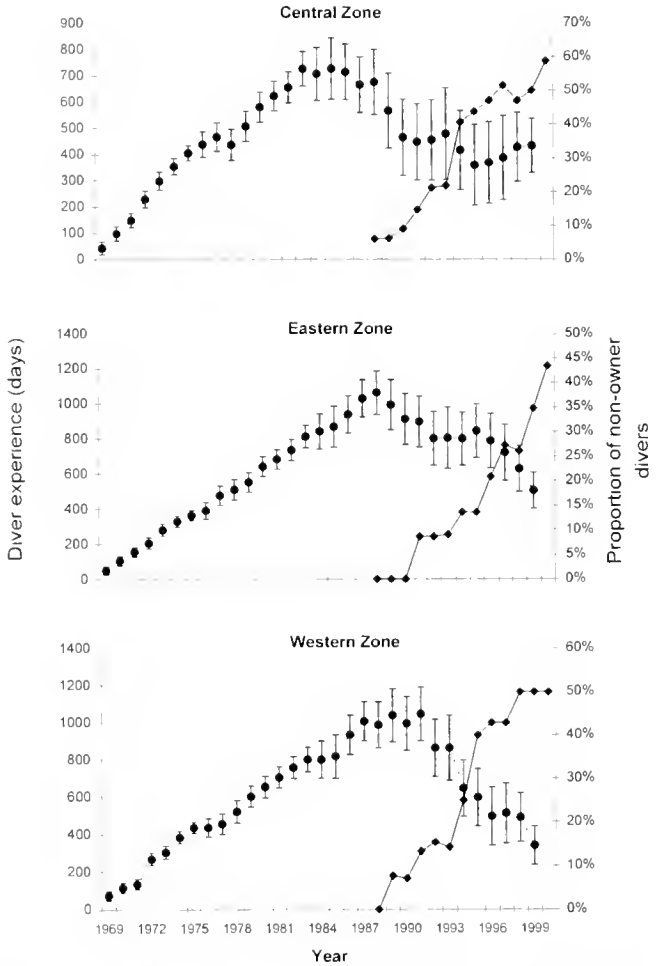

Figure 2. Changes in diver experience (mean ± SE; broken line) and the proportion of fishery access licences where the diver was not the license owner (solid line), in each of the three management zones during 1969–2000.

The latter two points describe an effective second tier of finer-scale self-management below the controls imposed by fisheries regulations. It is our belief that maintenance of this voluntary regime is both critical to the future sustainability of the resource and vulnerable to changes in industry structure that have a propensity to diminish stewardship.

With quotas comes the need to establish data collection programs that will support modeling and assessment of stocks for future TAC adjustment (Ramade-Villanueva et al. 1998). Diver-reported catch and effort statistics will generally be perceived as inadequate for this purpose (McShane 1992, although see Andrew et al. 1997). Like the limitations of quota management, this is partly a consequence of geographic scale that renders catch per unit effort data hyperstable to serial stock depletion (Breen 1992), although the abundance of dense aggregations of abalone also plays a role (Hart et al. 1997, Prince 1992). Inevitably, fishery-independent survey methods will be considered to provide the necessary data (McShane 1994). However, government-led independent surveys can be an expensive approach beset with the problems identified by Prince et al. (1998) about shrinking public sector resources in the face of the need to cover extensive fishing grounds.

In contrast with fishery-independent survey data, opportunities

to sample dependent data are more numerous, and their acquisition is cheaper (Kesteven 1997). This led us to speculate as to whether some of the impediments to using catch–effort data could be overcome if we considered divers as an integral part of the fishing gear. In trawl fisheries, for example, much data is collected about vessels, their fishing power, and fleet dynamics. Fisheries observer programs in southeastern Australia are well established as methods for collecting detailed information about trawl fishing fleets and their catches. Although the fishing power of divers has been studied in Australia (Beinssen 1979, Hart & Gorfine 1997), this has not been extended to monitoring programs involving fisheries observers. During late 1998, we initiated an on-board observer program directed at gaining a detailed understanding of the fleet dynamics, fishing effort, and contemporary fishing grounds of the Victorian abalone fishery (Gorfine et al. this publication). Although the quantity of data acquired to date is relatively small, these data provide information unavailable from catch and effort reporting. In this paper, we examine some descriptive statistics from our observer program and the insights they reveal about diver behavior. We draw comparisons with reported catch and effort statistics and then consider the implications of these behaviors for fishery management and stock assessment.

MATERIALS AND METHODS

Casual fisheries observers were employed at several major abalone-fishing ports along the Victorian coast. Each observer had contact information for licensed abalone divers in their vicinity who had agreed to participate in the program. The observers were instructed to spread their observations among as many divers and fishing locations as possible, because random sampling of the abalone fleet was impractical. Where possible, observers followed the catch through to processing to enable each day's observations to be linked to catch and effort information reported by divers and processors. A more extensive commercial catch-sampling program also operated in parallel with the observer program.

Observations made during each day aboard a particular diver's vessel comprised three levels of information:

1. An overview of the day's fishing, which included intended diving location, reason for its selection, catch expectation, type of abalone sought and reason why, details about the size, power, and hull style of the vessel, and weather and sea conditions;
2. GPS coordinates and divers' observations of the underwater habitat and environmental conditions, qualitative abundance of competitors and predators, and condition of abalone population as compared with previous visits at each dive location; and
3. Quantitative estimates of catch weight, number in the catch, and effort for each catch bag of abalone, qualitative estimates of the amount of shell epibiota and proportions of "tiger" variants and greenlip abalone, and the dive profile of the diver (depth recorded every 3 min by dive computer). Where possible, length frequency samples were taken from the day's catch after landing. However, to date length-frequency data have been collected too sporadically to analyze.

The three levels of information were entered into a relational database (MS Access®), and summary statistics were produced.

RESULTS

Summary

Six observers collected data from eighty-five dives over sixty-seven diver days. Observed catches totaled 32.3 tons live weight harvested by expending 215.6 h diver effort.

Vessels

Vessel length ranged from 5.6 to 8.5 m and engine power from 90–450 horsepower. Catamaran hulls were the most popular in the Western and Eastern Zones at 63 and 87%, respectively; however, Central Zone divers favored monohulls slightly, with only 40% catamarans.

Diving Practices

The maximum depth recorded out of 3,112 observations was 27 m, and the minimum was 1 m, with an average of 9 m and 95% confidence interval of 8.8–9.2 m. Percentiles of the distribution of depths show that 80% of all harvesting occurred shallower than 13 m or 43 ft (Table 1).

Diving practices ranged from lower to higher risk as demonstrated by recorded depth profiles (Fig. 3a). High-risk profiles involving progression to deeper depths later during the day were not necessarily associated with improved catch rates (Fig. 3b). Multiple ascents were a feature of most dive profiles recorded.

Site Selection

Of those occasions where divers gave specific reasons for site selection 50% related to weather, 38% to high catch expectation, 15% to diving safety, 12% to market requirements, and 4% to exploration of unfamiliar territory. Note that there were no constraints on the variety of reasons that could be recorded and that totals exceed 100% because of more than one reason given on some occasions.

Only one or two reefs were visited daily on 91% of the days observed during this study (Table 2). Although movement between reefs during a day's fishing was infrequent, divers often relocated within a reef complex, depending on their personal diving practices.

TABLE 1.

Quantiles of the distributions of diving depths and catch rates observed aboard commercial divers' vessels in the Victorian abalone fishery.

	Percentile										
	5	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**100**
Depth (m)	—	3	4	5	7	8	10	11	13	16	27
Catch rate (kgh^{-1})	65	79	106	120	133	150	165	183	204	240	386

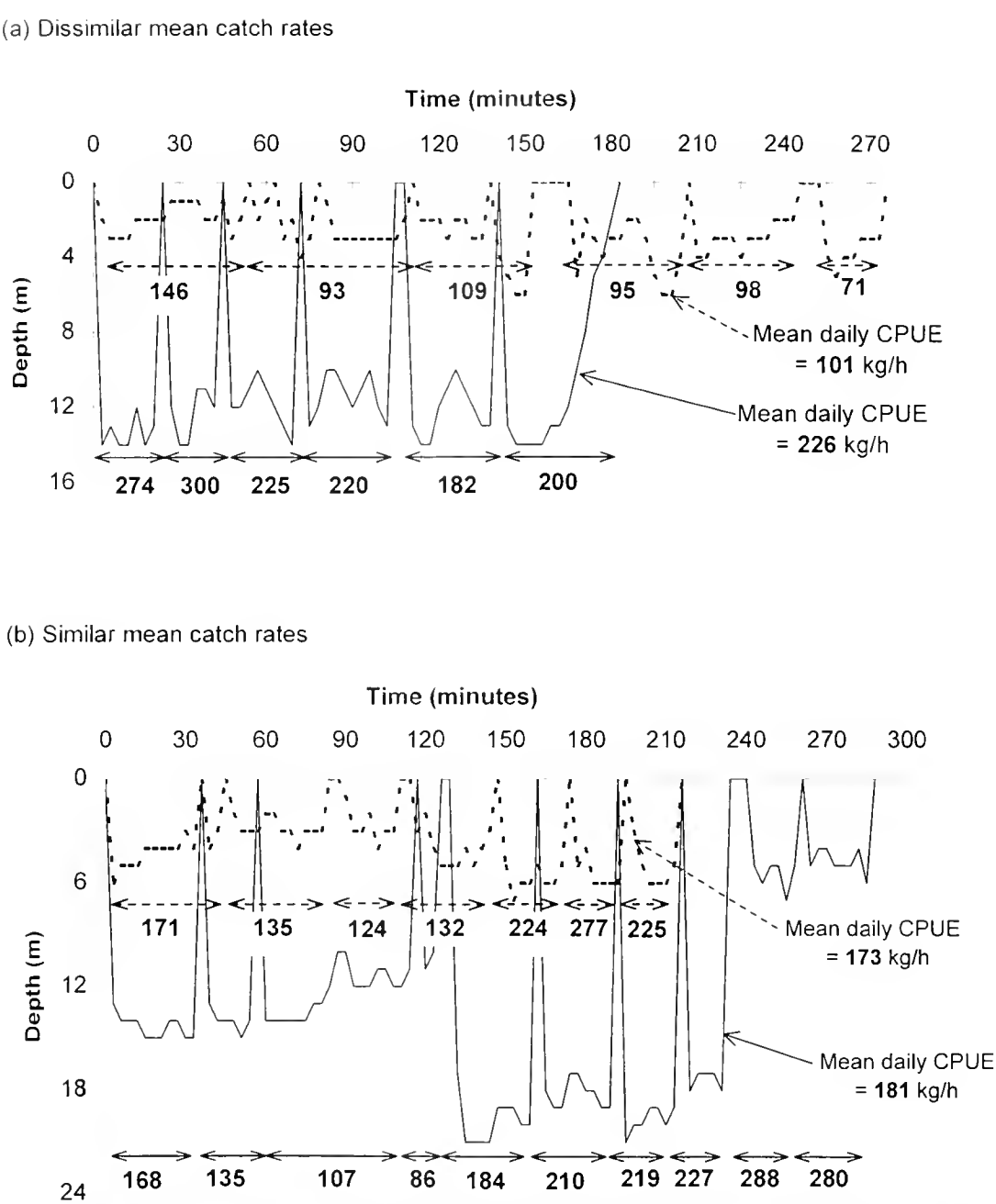

Figure 3. a) Different dive profiles yielding dissimilar mean daily catch rates (CPUE) for two divers in the same management zone of the Victorian abalone fishery. b) Different dive profiles yielding similar daily catch rates (CPUE) for two divers in the same management zone of the Victorian abalone fishery. (Broken line depicts low-risk dive profile and solid line depicts high-risk profile. Catch rates (kg/h) for each interval between surfacing are shown for each profile).

Harvesting Objectives

Divers reported that their principle objective for the day's fishing was large abalone on 28%, small abalone on 4%, and live abalone on 6% of occasions. Tigers; that is, a blacklip variant exhibiting a striped epidodium, were the target on 19% of occasions, usually in conjunction with small- or medium-sized abalone with the usual black epipodium.

Catch Expectation

The 95% confidence interval for daily catch expectation was 430–490 kg. The highest catch expectation was about 900 kg, and the lowest was 60 kg.

TABLE 2.

Frequency of number of reefs visited daily during on-board observations of the Victorian-abalone fishery.

	Number of Reefs Visited				
	1	**2**	**3**	**4**	**5**
Proportion of days (%)	55	36	5	2	2

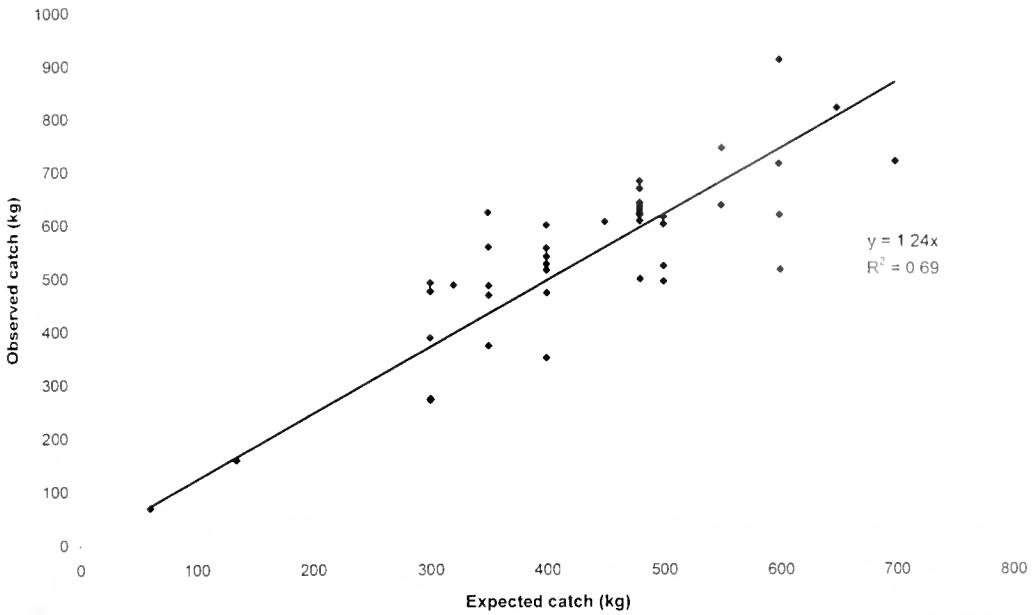

Figure 4. Relationship between expected and observed daily catches from observations on-board commercial divers' vessels in the Victorian abalone fishery.

Regression of observed catches on daily catch expectations showed that catches increased linearly with expectation (Fig. 4) and tended to exceed expected weights by 24% (df = 1, 41, r^2 = 0.69, t = 40.29, $P < 0.0001$). Comparison of expected, observed, and reported catches for a sample of the observations shows that reported catches were 8 ± 4% (mean ± SE) less than observed and expected catches were 15 ± 9% less than observed (Table 3). Only a small sample (n = 7) was available because of difficulties in matching observations with reported catches.

Catch Rates

Reef-specific catch rates ranged from 0–300 kgh^{-1} with 95% of values between 134–156 kgh^{-1}. Net bag catch rates ranged from 0–386 kgh^{-1} with 95% of values between 150–161 kgh^{-1} (Table 4). Reported effort values used to calculate catch per unit effort were 17 ± 9% higher than those we observed for the same catches (Table 3). Calculation of catch per unit effort (CPUE) using the mostly lower reported catch and lower reported effort gave values 12–29% (95% CL) less than the actual rates.

Although it is difficult to identify a threshold catch rate for the fishery explicitly, it is informative to consider that 80% of catch rates were greater than 106 kgh^{-1} (Table 1). The relatively small number of values below 50 kgh^{-1} (five observations) was for efforts of less than 20 min. Generally, there was no relationship between catch rates and duration of effort. However, unfavorable weather and low abundance accounted for the relatively few instances when catch rates were low. The former involved an ex-New South Wales diver new to the fishery, and the latter was associated with efforts of short duration. Maximum catch rates were associated with efforts of about 0.5 to 3.5 h, and longer efforts tended to produce slightly lower catch rates.

DISCUSSION

This modest study reinforces perceptions that commercial divers in the Victorian abalone fishery have developed fishing behaviors that maintain high catch rates enabling them to predict the quantity of abalone they expect to harvest from a specific area on a particular day reliably. To some extent, these patterns in behavior provide a degree of resource protection above that afforded by management regulations, because they tend to ensure that fishing mortality is generally low (McShane & Smith 1989).

TABLE 3.

Sample of divers' expected catches, estimated weights on landing and observed daily catch, effort, and CPUE, matched with reported catch, effort, and CPUE for the same observations.

Catch (kg)				Effort (min)		CPUE (kg/h^{-1})	
Expected	Divers' Estimate	Observed	Reported	Observed	Reported	Observed	Reported
—	550	567	539	293	480	116	67
400	350	474	344	121	120	235	172
300	230	273	232	219	240	75	58
350	400	470	414	160	150	176	166
350	300	378	368	179	210	127	105
600	400	517	476	166	180	187	159
350	450	527	585	170	220	186	160

TABLE 4.

Comparison of catch rate statistics at different scales of sampling during on-board observations of the Victorian abalone fishery.

Statistic	Scale of Sampling		
	Daily (kgh^{-1})	By Reef (kgh^{-1})	By Catch Bag (kgh^{-1})
Mean	144	156	156
Maximum	234	300	386
Minimum	7	0	0
Upper 95% CL	158	156	161
Lower 95% CL	131	134	150

Not surprisingly, the main harvesting objective of divers during this study was to maximize catch rates. However, on some occasions, abalone with striped epidodia (tigers) and smaller abalone were sought as the prime objective because of market preference. Smaller abalone, particularly from Port Phillip Bay, where the legal minimum length (LML) is 100 mm, are preferred for canning, because cans containing four or five whole pieces attract the best prices. The targeting of these specific morphotypes did not compromise divers' abilities to achieve average daily catches. Although daily catch expectations were mostly high, low catch expectations occurred when divers only required small quantities to obtain the balance of their quota allocations. Divers' catches exceeded their expectations in most instances. This is likely to have occurred because the divers base their catch expectations on weights used to decrement quota allocations. Consequently, we suspect that the divers have made allowances for losses attributable to the removal of epibiota and drainage during postharvest transport and storage (Gorfine this publication).

The faster catch rates observed at a within-reef spatial resolution highlight the loss of accuracy in effort associated with aggregating values at the reef scale. These inaccuracies are further amplified when catch rates are reported as total effort for the entire time spent at sea. When combined with post-harvest weight losses caused by removal of epibiota and drainage, it becomes evident why CPUE values extracted from Fisheries Victoria's catch and effort reporting system are often substantially lower than those measured directly during the course of this study.

Inspection of fisheries observers' comments noted on datasheets shows that the highest catch rates were for locations with very large abalone, each about 600–650 g live weight, that had large amounts of such shell epibiota as barnacles and sponges accounting for up to 30% of their weight. Allowing for removal of epibiota, the highest catch rate of 386 kgh^{-1} may have reduced to 270 kgh^{-1}. The amount and composition of epibiota generally reflects the particular habitat occupied by the abalone, with cunjevoi adding the most weight, and divers inform us that they believe abalone with clean shells are usually those that have recently emerged from cryptic habitat. Previous studies we have undertaken at a limited number of sites have not supported this hypothesis (Officer et al. this publication).

Weather played a dominant role in divers' decisions about where to fish. Generally, most Victorian divers do not head to sea if weather conditions are likely to prevent them from satisfying their catch expectations. This is reflected in some of our fisheries observers' experiences of aborted trips despite preplanning and weather forecasting. Examination of the relationship between effort and catch rate showed that a small number of low catch rates for large effort expenditure related to weather, lack of experience, or a combination of both these factors. Indeed, one of the divers had only recently commenced in the Victorian fishery after having fished in NSW where maximum catch rates are typically about 25 kgh^{-1} (Worthington et al. 1999). Although an experienced and competent diver, he may have been prepared to fish at lower catch rates than most Victorian divers would tolerate, because the 70 kgh^{-1} he achieved was more than double the rate he would have experienced in the NSW fishery.

The vessels used by Victorian divers reflect a combination of geography and economics. In some instances, individual divers may own different vessels to access different parts of the coast. Monohulls are preferred where on-road towing distances are long and at-sea distances short and where beach launching across sand is required. This applies more to Central Zone divers than to those from the other zones of the fishery. The lower operating costs of monohulls as compared to catamaran hulls also make them attractive to contract divers. Twin hulls are preferred where bar crossings are made and where long distances on the water are necessary in sometimes fickle weather conditions, such as in the Eastern and Western Zones. As more contract divers participate in the fishery, we may see an increase in the number of monohulls. To some extent, this may limit the intensity of fishing at remote locations and concentrate effort in relatively safer areas closer to ports of access.

Despite the consistency in high catch rates among divers, there is considerable variability in diving practices. A small number of divers spend a large proportion of their time continuously underwater, preferring to send their catch bags of abalone to the surface using inflated parachute-style lift bags. These divers ascend infrequently; whereas, others make frequent multiple ascents, sometimes from relatively deep depths. Some divers use large catch bags with a capacity of about 150 kg; whereas, others use small bags that hold about 50 kg. Time spent transporting the catch across the substrate and to the surface is counted as effort, and the size of the catch bag could reasonably be expected to influence catch rates.

Two divers reported that they only dived to shallow depths. One diver never exceeds 10 m; whereas, the other, who suffered two episodes of decompression illness (DCI) during 1998, does not exceed 12 m, in accordance with medical advice. Although these divers believe their practices are conservative, examination of their profiles shows an ignorance of the effects of multiple ascents in precipitating DCI (Fig. 3a). The trade-off for these "conservative" profiles was reduced catch rates in some instances but not in others, so it does not necessarily follow that diving to deeper depths will yield better catches. In contrast, another diver showed a willingness to defy accepted wisdom by diving deeper on successive dives in an attempt to maximize his catch rates (Fig. 3b). Ironically, it was during a shallow "decompression" dive toward the end of the day that he achieved his best results.

Although diving depth may be unrelated to the ability to satisfy catch expectations, at present it may become a factor if stocks in shallower locations decline because of increased intensity of effort. The relatively shallow depths of most of the diving observed during this study reflect a tendency for younger contract divers to adopt safer practices and concentrate their effort on shallower reefs. In recent instances where localized stock depletion has oc-

curred (Gorfine et al. this publication), divers have relocated effort previously expended on the depleted reefs to other regularly fished reefs in their zone. There is no evidence that divers have shifted their effort offshore to those deeper or more remote reefs that have been infrequently fished since quotas were introduced.

This study has illustrated some aspects of diver behavior that may have substantial influence on the state of Victorian abalone resources and the long-term sustainability of the fishery. One of the key observations was the high, but variable, catch rate thresholds that prompted divers to shift their effort within a reef complex. Net reductions in these thresholds over time will signal overfishing of the most productive reefs. It is likely that such changes will lead to an over-all decrease in the performance of the fishery, because underutilized reefs where effort can be redeployed are mostly those with historically low catch rates.

Departures from established such practices as self-imposed daily quotas and rotational harvesting will threaten abalone stocks regardless of current zonal quotas and regional size limits. This is not merely speculative, given that the fishery is progressively shifting to a new generation of contract divers (Fig. 2) motivated by different values, past experiences, and expectations that will be reflected in their fishing behaviors. Although many contract divers seem to be genuinely interested in the on-going viability of the fishery, they do not necessarily have the same incentive as quota-owning divers to ensure that their fishing practices are compatible with long-term sustainability (Prince & Shepherd 1992, Parliament of Victoria 2000). Victorian abalone divers are understandably proud of their history of resource stewardship and the altruism they displayed in reducing their catches in the shift to quota-based management twelve years ago. In a brief discussion on the evolution of altruism, noted ecologist Simon Levin (1999) points out that altruistic behavior is the manifestation of "enlightened self-interest" and is strongest when there are expectations of rapid payoffs to individuals or coalitions sharing common aspirations. Obviously, the self-interests of divers who are either quota owners or close relatives likely to inherit some proportion of quota entitlement will differ from those without equity in quota. The proportion of the beach price (usually less than 10%) received by contract divers from the owners of fishery access licenses is sufficiently small to ensure that the contract divers are likely to be more concerned with reducing the costs of fishing effort to maximize their incomes. In an assessment of the Tasmanian abalone fishery, where there is clear separation between quota ownership and the licensing of divers, Officer (1999) expressed similar concerns that contract divers reluctant to incur the costs of travel to remote locations may be prepared to endure lower catch rates than owner-divers on more accessible reefs.

Currently, those charged with developing a management plan for the Victorian fishery are proposing changes toward a system similar to the one adopted in Tasmania. These changes will facilitate further separation between quota ownership and fishery access licenses that confer the right to dive or engage a contractor to dive for abalone. Whether additional management arrangements, such as small-scale subzonal quotas, are required to modify potential changes in diver behavior to be within acceptable limits is unclear.

At this stage, there is no evidence that the over-all status of Victorian abalone stocks has been compromised by changes in the licensed catching sector. However, there is clear evidence that the fishing grounds have contracted substantially since quota introduction and that the fishery is now dependent on a limited number of relatively shallow and highly productive reefs (Gorfine et al. this publication). There is no doubt that this makes the Victorian abalone fishery more vulnerable to the impact of serial depletion and threatens its sustainability. We believe it would be preferable for industry to remain self-regulating at finer spatial scales, but this view is contingent on the Victorian abalone industry's ability to maintain the resource stewardship characteristic of the retiring generation of owner-divers. The concept of a Territorial Users' Rights Fishery (TURF) espoused by Prince et al. (1998), with potential benefits of fine-scale monitoring and industry self-governance, could be an effective framework for such stewardship. However, the public acceptance of this kind of scheme would be improbable for a resource that is the common property of the whole community of Victoria.

As fisheries management agencies in the Northern Hemisphere look toward quota management as a means of improving the sustainability of their abalone fisheries they should be cognizant of the limitations of this approach, particularly for a fishery in need of restoration. Although quotas may prove to be the best option, how they are applied as part of a suite of management arrangements will be critical for their effectiveness. Abalone fisheries managers must be mindful that regulations affect diver behavior and that principally it is the divers, not the resource, that is managed. We believe that this study highlights the importance of identifying and promoting diver behaviors that are compatible with resource sustainability within the context of contemporary management strategies. Such fisheries observer programs as ours can provide valuable information to managers for achieving this objective.

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POST-HARVEST WEIGHT LOSS HAS IMPORTANT IMPLICATIONS FOR ABALONE QUOTA MANAGEMENT

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ABSTRACT Regulations forming part of the abalone quota management system in Victoria, Australia require that abalone processors notify fisheries authorities of quantities of abalone consigned from commercial divers within 25 h of landing. The regulations also require that the abalone are to be landed whole in the shell, and transported and stored in sealed bins until confirmation of official notification. Although the bins cannot be opened before notification, the 25-h limit for notification provides a window of opportunity to make potential gains through weight loss in those abalone destined for canning. This arises because notified weights, rather than weights estimated upon landing, are used to decrement the quota allocations of individual divers. I investigated the potential for post-harvest weight loss by subjecting abalone to three experimental treatments selected to simulate a range of possible transport and storage regimes. My results demonstrate that substantial weight loss can occur in whole abalone during both ambient and refrigerated storage. Loss of weight in abalone during storage results from the release of water and body fluid associated with physiological responses to hypoxic stress caused by exposure to air. In Victoria, about 60% of the landed catch is used to produce canned product. Because of the observed weight losses during storage, divers supplying abalone for canning have to harvest more abalone to achieve their quotas than those supplying abalone for live export. Losses in weight of 10–20% observed during this study equate to a harvest of 6–12% (350,000–700,000 abalone) more than the total allowable catches. Harvesting these additional quantities of abalone may exceed desired fishing mortalities for long-term population sustainability. If beach weights were deducted from quotas, then this problem could be resolved.

KEY WORDS: quotas, management, weight loss, air exposure, abalone

INTRODUCTION

The Victorian fishery for blacklip abalone (*Haliotis rubra* Leach) has operated under quota management since 1988. Total allowable catches (TACs) were initially based on average annual catches during the preceding 5 y. A quota docketing system was implemented concurrently to track divers' progress in attaining their annual TACs. It became apparent during the early 1990s that some processors were taking advantage of regulations that permitted a transportation period of up to 1 day post-harvest before the consignment of catches and completion of quota dockets. Potential fluid loss during this period enabled some processors to pay less for the same number of abalone. Correspondence from this period shows that some of the larger processors and diver associations sought to have this loophole in the regulations removed.

During 1994 additional regulations were proposed to ensure that, immediately upon landing, abalone would be placed in bins sealed with nonreusable identification tags and weighed at the beach. The principal objective was to eliminate the potential for overquota harvesting through understatement of estimated catches on quota dockets. Sealed bins prevent the removal of abalone between the point of landing and delivery to registered abalone processing or storage premises. Beach weighing ensures that processor weights can be reconciled with landed weights. New regulations requiring the sealing of bins and establishing a comprehensive audit trail were introduced during 1996. However, attempts to introduce beach weighing for decrementing quota allocations were unsuccessful. This was largely because processors close to points of landing argued that it was an unnecessary imposition when their factories were only about 10 min away. Ironically, processors distant from points of landing, and the divers supplying these processors, lobbied for sufficient time before notification to allow abalone harvested at one end of the state to be transported to a processor at the other end (a distance of almost 900 km). Such arguments by the abalone industry tend to be successful because of the Australian federal government's National Competition Policy legislation, which, in seeking to remove barriers to business and trade, requires any commercially restrictive regulations to be justified on resource protection grounds.

The 1996 regulations, forming part of the Abalone Quota Management System, require abalone processors to notify Fisheries Victoria of quantities of abalone (as weights to the nearest 0.1 kg) consigned from commercial divers within 25 h of landing. This is done via electronic facsimile to an automated notification service that then provides confirmation by return transmission within 15 min of receipt. Processors must not break the seals on the bins until this confirmation is received. The particular diver's quota is then decremented accordingly. The current practice among many abalone processors is to take possession of abalone during the afternoon or evening on the day of landing, store them in a cool room overnight, and not effect consignment and notification until early the following morning before processing. This means the abalone may be emersed for 12–25 h before weighing.

Despite the paucity of literature on the effects of emersion on haliotids, there is a substantial body of work on intertidal gastropods, including prosobranchs. Many intertidal prosobranchs have adapted to survive extensive periods of hypoxic stress and desiccation associated with exposure to air, as well as temperatures beyond the range experienced during immersion. Physical adaptive strategies include a relatively large water storage capacity within the mantle cavity (Hyman 1967), a shell morphology that provides a large volume to surface area ratio (Newell 1979), capacity to key the irregularities of the shell margin to fit the perimeter of the homesite occupied closely (Hyman 1967), closure of the shell aperture with opercula (Newell 1979), and vertical orientation (Hyman 1967). Physiological strategies include enhanced osmoregulatory ability (Gardiner 1972), excretion of uric acid rather than ammonia compounds to conserve water (Hickman 1973), uptake of seawater into the gland cavities of the foot, remaining motionless while exposed, and acclimation by lowering the meta-

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bolic rate to reduce oxygen consumption (Hyman 1967). Among all these strategies, water-holding capacity is the most important, because it prevents desiccation of the gill tissues, provides limited oxygen for metabolic activity, reduces thermal stress through evaporative heat loss, and dilutes otherwise lethal concentrations of solutes in body fluids (Newell 1979).

Although they are mostly subtidal, air-exposed blacklip abalone have been observed under vertical overhangs immediately above the low-water mark and may possess some of the adaptations of predominantly intertidal species from the same order. Other species of abalone are more frequently exposed to air in the lower intertidal. For example, the tropical species *Haliotis asinina* (Linné) forages atop coral heads exposed at low tide (Donovan et al. 1999), and some populations of *Haliotis rufescens* (Swainson) may be exposed to air for up to 1 h during normal tidal cycles (Tjeerdema et al. 1991). This may explain why blacklip abalone are able to retain water after removal from the sea; furthermore, it may explain the pattern of progressive water loss while these abalone attempt to survive extended periods of air exposure. Survival time is likely to affect the rate of water and body fluid loss, and abalone biochemistry shows some adaptation in glycolytic pathways that promotes survival of hypoxia during air exposure (Donovan et al. 1999, Wells & Baldwin 1995).

The present study aims to measure the weight loss associated with up to 25 h emersion by simulating conditions typically encountered at abalone processing facilities. I was originally requested to investigate this potential for weight loss during storage in 1994, because some sectors of industry had indicated they would seek compensation if beach weighing was introduced. My initial research demonstrated that industry claims regarding post-harvest weight loss could be substantiated. However, recent development of a fishery model, aimed at establishing the risks to sustainability of different TACs, created the need for additional research to confirm the initial results and enable more accurate catches to be input to the model.

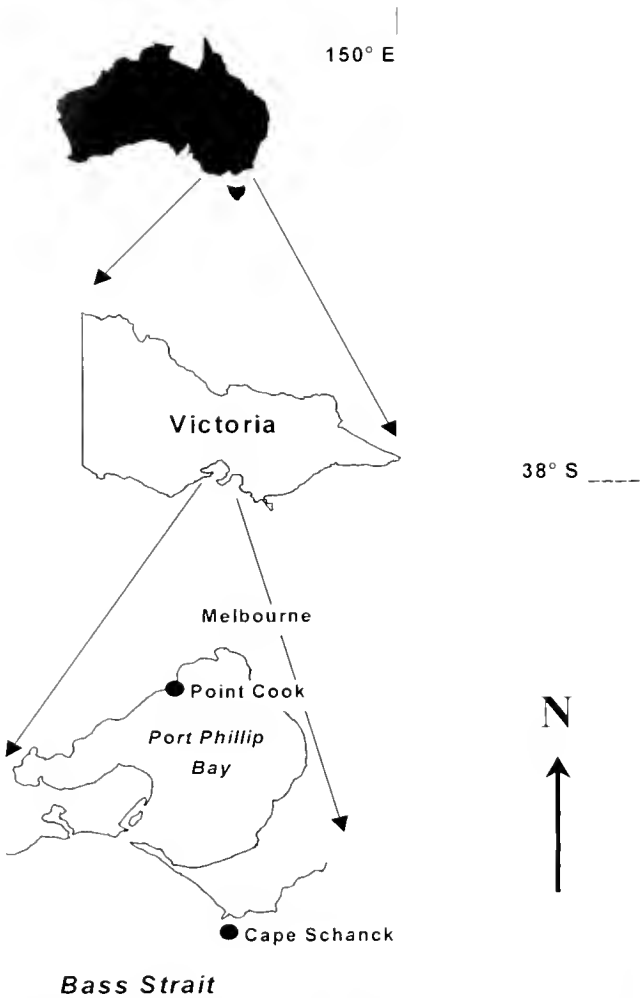

Figure 1. Locations of blacklip abalone (*H. rubra* Leach) collection sites along the coast of Victoria, Australia.

MATERIALS AND METHODS

Two experiments were conducted to investigate post-harvest weight loss in air-exposed blacklip abalone (*H. rubra*) during storage. The first experiment measured losses through drainage from commercially used bins of abalone, and the second experiment measured fluid loss from individually contained abalone.

Experiment 1: Weight Loss from Commercial Bins of Abalone

In warm to hot weather between 0930 h and 1330 h during December 1994, research divers harvested 300 kg whole weight of abalone from Bushranger's Bay near Cape Schanck, Victoria (Fig. 1). The majority of these abalone were marginally larger than the legal minimum length of 110 mm and did not seem to have recently spawned. The abalone were packed into prelabeled Nallys® No.12 fish bins in a primary layer consisting of three longitudinal rows of abalone oriented vertically and a secondary layer placed in a horizontal plane. The total weight for each bin was about 20 kg. The bases of the bins were perforated to allow for the drainage of water and abalone body fluids. Abalone harvested at different times were equally distributed among experimental treatments, and all abalone were landed at 1445 h when initial weights were measured to the nearest 0.05 kg. Each bin was reweighed every half-hour for a period of 5 h, and final weights were determined after an additional 12 h.

Three experimental treatments were applied to sets of five replicate bins of abalone:

1. *cool*—involved refrigeration at 6°C immediately post landing;
2. *ambient*—required the abalone to remain uncovered at ambient temperature (24.5°C @ 1445 h to 22°C @ 2000 h) for 5 h before refrigeration at 6°C for a further 12 h; and
3. *moist*—the abalone were covered with moist cloth towels at ambient temperature (24.5°C @ 1445 h to 22°C @ 2000 h) for 5 h before refrigeration at 6°C for a further 12 h.

Treatments 2 and 3 were conducted under covered walkways, so the bins of abalone were not exposed to direct sunlight. The air temperature at 1200 h in direct sunlight was close to 30°C. Relative humidity was not measured.

Experiment 2: Weight Loss from Individual Abalone

This experiment was conducted on a warm day (26°C at 1200 h in direct sunlight) during November 1999. Two storage environments were used, an air-conditioned laboratory in which temperature was maintained in the low 20s (°C) and a cool room with the thermostat set at 3°C. Abalone ranging in shell length from 83–120 mm were collected from Point Cook in Port Phillip Bay (Fig. 1) and held in one ton capacity, flow-through aquaria for several

months before this experiment. Abalone were removed from the aquaria, blotted on absorbent towels to remove excess external seawater, individually placed in preweighed 1.0-L plastic containers, and weighed to the nearest 0.1 g. Orientation of abalone in the containers was, where possible, in a vertical plane with the leading shell margin facing downward. Each container was fitted with a plastic grill to allow water to drain away from the abalone. Forty-five abalone were randomly assigned to each of the following three storage treatments:

1. *cool*—involved refrigeration at 3°C immediately after weighing;
2. *amcool*—required the abalone to remain at ambient temperature (24°C @ 1400 h) for 4 h before refrigeration (3°C @ 2000 h) for a further 20 h; and
3. *ambient*—the abalone remained at ambient temperature (24°C @ 1400 h to 20°C @ 2000 h) for 24 h.

Abalone were reweighed in their containers after 1, 2, 4, 6, 8, 10, and 24 h. At each weighing, the combined weight of the container, abalone, and drained abalone fluid was measured. Then the weight of the container and fluid with the abalone lifted above, but not out of, the container was measured. As the abalone were weighed, observations were made regarding the degree of pedal adhesion and responsiveness to stimulation.

Although relative humidity was not measured directly, evaporative loss of water was used as a *de facto* alternative. To test for evaporative loss of water 10 1.0-L plastic containers filled with about 350-mL tapwater were placed in each of the two storage environments and reweighed at the same time intervals as the abalone, and additionally at 5 h.

Potential for fluid loss was investigated by measuring the volume and weight of fluid that could be drained by gentle squeezing of the soft tissues of fifty abalone selected at random from the same aquarium.

Statistical Analysis

Weight loss in the first experiment was determined from the difference between the combined weight of the bin and abalone at t_i and the initial weight at t_0. In the second experiment, weight loss caused by evaporation and handling was calculated as the difference between the weight of each abalone in its container at time t_i and at the commencement of the experiment at t_0. The differences between the weight of an abalone in its container at t_i, the weight of the container with the abalone suspended above it at t_i and the weight of the empty container at t_0 were used to determine the weight of fluid lost directly through physiological processes.

For both experiments, the weight of fluid lost was then subject to analysis of variance using the ANOVA procedure in the SAS® statistical software package (SAS Institute 1990). Data from Experiment 2 were log (x + 1) transformed to reduced heterogeneity of variances among treatment combinations, eliminate a positive linear relationship between treatment means and their variances, and reduce non-normality in their distribution.

The ANOVA tested weight loss as a response to the predictor variables of storage exposure (Treatment) and duration of exposure (Time). A two-factor ANOVA model with repeated measures on Time was specified. Because, in all instances, the interaction between storage treatment and time was highly significant ($P < 0.01$) simple investigations of levels of main effects were conducted. One-way ANOVA was performed on storage treatments for each level of time and then repeated measures one-way ANOVA on time was applied to each storage treatment. The Scheffé test for multiple comparison of means was used to detect significant differences among levels of each predictor variable. Volume of fluid lost by squeezing was regressed against the weight of this fluid for 50 additional abalone.

RESULTS

Experiment 1: Weight Loss from Bins of Abalone

Significant weight loss (df = 10,120, F = 3.04, $P = 0.0001$), ranging from 8–14%, occurred through drainage from bins of abalone over 17 h. Although there were no significant differences among treatments (df = 2,12, F = 0.07, $P = 0.5168$), probably because of low statistical power (Table 1), continuous storage in the cool room produced the largest loss; whereas, covering with a moist cloth at ambient temperatures produced the least (Fig. 2). Further analysis showed that weight loss increased significantly over time for all three treatments (df = 10,40, $F_{Cool} = 41.33$, $F_{Ambient} = 24.46$, $F_{Moist} = 5.89$, $P = 0.0001$). Multiple comparison of means (Table 2) showed that weight loss of abalone stored under cool conditions was not significant over the first 5 h but was significantly greater between 5 and 17 h. Weight loss under ambient conditions differed significantly over time intervals greater than 3 h throughout the experiment. There were significant differences between the weight loss during the first 1.5 h and losses over the subsequent 15.5 h. The lack of significant contrast in the means is almost certainly a result of the low power of the analysis. Minimum significant differences (MSDs) over time were greater than 50% of the grand mean for all treatments (Table 1). The low power was a consequence of the use of only five replicates per treatment under circumstances where differential drainage through holes in the bases of the plastic bins caused substantial within-treatment variation.

Experiment 2: Weight Loss from Individual Abalone

Fluid loss from individual abalone varied significantly between cool (3°C) and ambient (21°C) storage (df = 2,132, F = 6.16, $P = 0.0028$) and over time (df = 6,292, F = 905.17, $P = 0.0001$). After 24 h, fluid loss from ambient (15% loss) and cool (14% loss) did not differ significantly, but fluid loss from those abalone transferred from ambient to cool conditions (20% loss) was significantly greater than in the other two treatments (Table 3). The

TABLE 1.

Minimum significant differences (MSD) in weight loss from bins of abalone, among treatment means and as a proportion of the grand mean for each time level ($\alpha = 0.05$).

Time (h)	Grand Mean	MSD	MSD/Grand Mean (%)
0.5	0.92	1.48	161
1.0	1.53	2.33	152
1.5	2.02	2.83	140
2.0	2.59	2.91	112
2.5	2.96	3.06	103
3.0	3.54	3.29	93
3.5	3.97	3.54	89
4.0	4.43	3.84	87
4.5	5.02	4.32	86
5.0	5.30	4.29	81
17.0	10.83	8.15	75

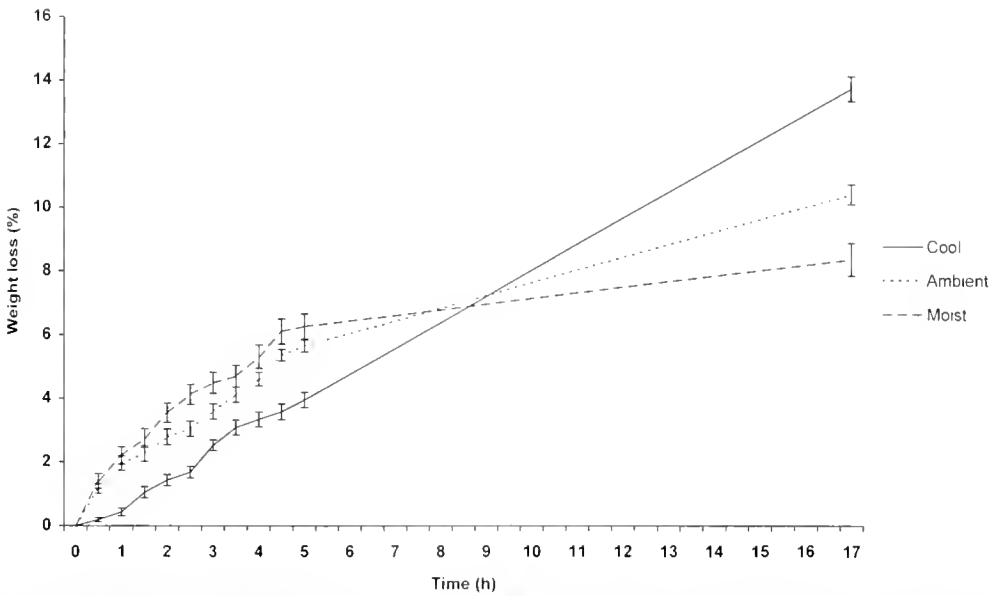

Figure 2. Mean cumulative weight loss (%) over time for commercial sized bins of blacklip abalone (*H. rubra*) subjected to three different conditions of storage (error bars are standard errors).

largest difference between cool and ambient treatments occurred after 4–6 h, and the greatest difference among all three treatments occurred at 8 h (Fig. 3). Most fluid loss occurred during the first 6–8 h of exposure (Table 4). Abalone with the most weight loss at each time of measurement tended to have the poorest pedal adhesion, consequently strong pedal adhesion was least apparent among abalone undergoing cool storage.

Differences in evaporative loss of tapwater between cool and ambient temperatures (df = 1,18, F = 8.61, $P = 0.0089$) and over time (df = 7,126, F = 137.95, $P = 0.0001$) were significant but small (<2% of treatment means). For each treatment, the losses were relatively small, ranging from 1.4–1.5% (95% confidence) in the cool room and 1.5–1.7% (95% confidence) in the ambient room after 24 h. Evaporation under ambient conditions was initially less than in the cool room during the first 4 h (Table 5). After 4 h had elapsed, the two curves converged when the ambient temperature rose to a maximum for the experiment of 24°C (Fig. 4). From 4–10 h, there was no significant difference between treatments and after 24 h, ambient evaporation was slightly greater than in cool room (Table 5a). Evaporative loss occurred significantly throughout the time period, although the difference in loss between 5 and 6 h under ambient conditions was nonsignificant (Table 5b).

Evaporative losses of the more viscous abalone fluid from the containers in which the abalone were housed over the same period were somewhat greater than the tapwater evaporation. The 95% confidence ranges for the evaporation of abalone fluid were 2.2–2.4% for cool, 4.2–4.4% for ambient and 3.0–3.3% for abalone transferred from ambient to cool.

Potential for loss by squeezing abalone showed fluid contents ranging from less than 1–15%, with 95% of individuals containing between 5–7% of fluid. Regression of volume versus weight of fluid showed close correspondence ($n = 50$, $R^2 = 0.92$) sufficient to assume 1.0 g ≡ 1.0 mL fluid.

DISCUSSION

Exposure of prosobranch gastropods to air occurs naturally for those living in the intertidal zone. The greater the height of exis-

TABLE 2.

Scheffé multiple comparison of differences among mean weight losses from bins of abalone over time for each treatment level (underlining bar ——— identifies means that were not significantly different, α = 0.05).

	Time (h)										
Treatment	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**	**4.5**	**5.0**	**17.0**
Cool	0.19	0.43	1.05	1.44	1.69	2.53	3.09	3.35	3.59	3.96	13.73
Ambient	1.17	1.96	2.26	2.79	3.06	3.60	4.13	4.62	5.37	5.66	10.41
Moist	1.41	2.20	2.75	3.55	4.13	4.50	4.71	5.33	6.11	6.28	8.36

TABLE 3.

Simple comparison of treatment means of fluid loss from individual abalone exposed to air for each time level ($\alpha = 0.05$; data were log (x + 1) transformed).

Time (h)	df	F	*P*	Scheffé Comparison	Grand Mean	MSD
1	2,132	1.83	0.1638	Nonsignificant	1.40	0.22
2	2,132	2.29	0.1048	Nonsignificant	1.58	0.23
4	2,132	18.27	0.0001	Amcool ≡ ambient < cool	2.04	0.25
6	2,132	19.11	0.0001	Ambient < cool ≡ amcool	2.55	0.24
8	2,132	41.27	0.0001	Ambient < cool < amcool	2.77	0.20
10	2,132	32.42	0.0001	Ambient < cool < amcool	2.84	0.20
24	2,132	12.14	0.0001	Cool ≡ ambient < amcool	3.05	0.17

tence above the low-water mark, the greater the adaptation to prolonged air exposure. Resistance to desiccation is of prime importance to these intertidal marine snails, and, in the extreme, some species can survive in their natural habitat for months without seawater contact. Loss of body water is the direct cause of death from desiccation and losses within the range of 10–30% water content have resulted in mortality in several species (Hyman 1967). Abalone are predominantly subtidal and are, therefore, less adapted to survive desiccation, hypoxia, and thermal stress from prolonged air exposure. Although the abalone mantle cavity has a moderate water storage capacity, the ability to retain that water during emersion is limited by having a large shell aperture. My results show substantial loss of water and body fluid through drainage from the mantle cavity within 24 h of air exposure. Newell (1979) provides an example where water loss over time at 21°C for the lower shore limpet *Patella cochlear* ranged between 25–35% at 5 h and 35–40% after 50 h. These ranges of water loss are about twice that of the second experiment in my study.

Losses of 5–20% were sufficient to cause substantial morbidity and mortality within the first 10 h and almost 100% mortality after 24 h with some notable exceptions. A couple of large abalone held at ambient temperature seemed exceptionally healthy at the conclusion of the second experiment. Newell (1979) cites a study of six species of intertidal prosobranchs from the Cape Peninsula, South Africa, that showed median mortality ranged from 16–33% among species, depending on the period of emersion and duration of exposure to low relative humidity. Time to 50% mortality ranged from 1–12 days at temperatures between 3.5–20 C with generally shorter survival at the higher temperatures. As stored water is lost from the mantle cavity and body tissues, thermal tolerance will diminish and osmotic concentration of body fluid may tend toward lethal levels (Newell 1979).

The larger percentage of losses in the second experiment as compared with the first possibly arose because of drainage losses from bins occurring aboard the research vessel while at sea during the first experiment. These losses were not measured; whereas, all losses during the second experiment were accounted for in the estimates. Another plausible reason for the difference between the two experiments may have been retention of lost fluid in the first experiment that failed to drain through the bases of the bins during the postlanding period. The retained fluid would have been included in the weights of the bins of abalone. The high initial MSDs relative to the mean weights during the first experiment possibly reflect within-treatment variability in the amount of water retained in each bin. This variability is likely to have reduced over time, because movement of bins during repeated weighing caused pro-

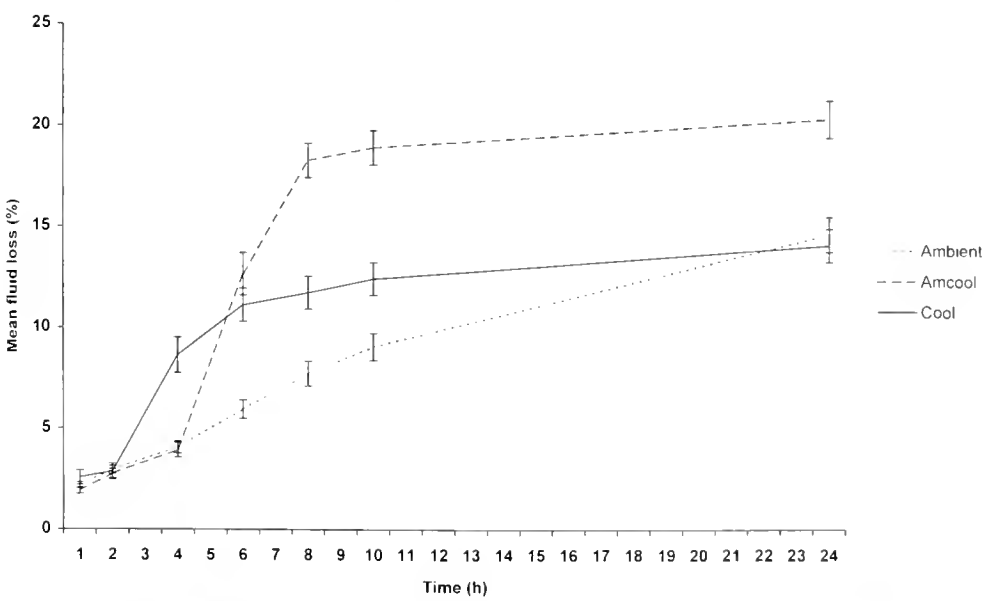

Figure 3. Mean cumulative fluid loss (%) over time for individual blacklip abalone (*H. rubra*) subjected to three different storage conditions (error bars are standard errors).

TABLE 4.

Simple comparison of differences in fluid loss from individual abalone exposed to air over time for each treatment level ($\alpha = 0.05$; data were log (x + 1) transformed).

Treatment	df	F	*P*	Scheffé Comparison	Grand Mean	MSD
Ambient	6,264	261.39	0.0001	1h < 2h < 4h < 6h < 8h ≡ 10h ≡ 24h	2.18	0.16
Amcool	6,264	434.39	0.0001	1h < 2h < 4h < 6h < 8h ≡ 10h ≡ 24h	2.43	0.20
Cool	6,264	264.45	0.0001	1h ≡ 2h < 4h < 6h ≡ 8h ≡ 10h ≡ 24h, & 6 < 24	2.34	0.26

gressive loss of retained water. In other words, the statistical power to detect significant weight loss increased during the course of the experiment. Although the use of bins accurately portrays industry practice, the use of individual containers more accurately reflects the potential for weight loss.

There is some evidence that contraction of the pedal muscle is associated with water loss. Hyman (1967) cites a study by Morris showing that when the pedal muscle of *Nactica* species is stimulated to contract, fluid is emitted from the mantle cavity in a volume similar to the capacity of the shell. The initial rapid increase in weight loss when abalone were transferred to the cool room may have arisen from pedal muscle activity that caused more rapid water loss than occurred in those abalone stored under ambient conditions. Poor pedal adhesion, particularly among those abalone subjected to cool storage conditions, may have been related to water loss from the pedal muscle, or reduced activity levels to conserve energy. Alternatively, it may have been a consequence of a hypoxia-induced reduction in muscle tone. Failure to maintain strong adhesion to the substrate is a clear sign of stress or morbidity in abalone. Under normal conditions, large abalone have been observed to resist a force of one-half ton (Hyman 1967).

The shell morphology of abalone is also less suited to thermal stress during air exposure than the shells of more globose-shaped prosobranchs, such as the turbinids and trochids, that have the additional advantage of an operculum. Temperature is the single most important factor in controlling the quality and shelf life of fresh seafood. Consequently, it is usual for most seafood to be stored at low temperatures before processing. Both bacterial growth and biochemical reactions will be increased when harvested abalone are stored at elevated temperatures. Although cold storage will suppress increases in hosted bacterial populations, paradoxically, it may depress biochemical processes that otherwise defray the negative effects of hypoxic stress due to air exposure. Abalone of several species subjected to hypoxic stress caused by air exposure have been shown to produce increased quantities of the glycolytic metabolites D-lactate and tauropine rapidly, and to use arginine phosphate as an additional source of anaerobic energy (Donovan et al. 1999, Tjeerdema et al. 1991, Watanabe et al. 1992, Wells & Baldwin 1995). Wells and Baldwin (1995) refer to speculation that the accumulation of tauropine as an additional pyruvate reductase end-product may be an adaptation in haliotids to protect their tissues during hypoxic episodes. Their studies indicate that lactate is the preferred product during such environmental hypoxia as air exposure and tauropine during functional hypoxia arising from exercise. They suggest that abalone may be adapted to continue metabolizing during air exposure associated with commercial handling, processing, and shipping, despite an inability to irrigate their gills. Unlike my results, most of the abalone (*H. iris* Martyn and *H. australis* Gmelin) exposed to air during their study maintained good pedal adhesion and muscle tone for 24 h. The capacity to use anaerobic glycolysis during hypoxia is likely to vary among abalone species (Donovan et al. 1999). It is possible that *H. rubra* is more dependent on aerobic metabolism than species studied to date, but this demands further investigation. The greater weight loss and apparently higher morbidity in abalone initially exposed to ambient air for several hours before refrigerated storage may be related to disruption of these biochemical processes caused by substantial reductions in temperature.

This may partly explain why abalone held at ambient temperature lost less fluid than those placed directly in cold storage (<5°C). However, the poorer performance of those transferred to cold storage after 4 h seems likely to have resulted from the additional stress imposed by multiple temperature changes. The variability in differences among treatments over time may reflect the effects of thermal stress interfering with biochemical responses to hypoxic stress.

Although I initially suspected that fluid loss may involve active physiological processes directed at preventing gill desiccation, this does not seem to have extended to active excretion of water to irrigate the gills, as has been reported for limpets (G. Parry pers.

TABLE 5(a).

Simple comparison of tapwater evaporation (%) treatment means for each time level ($\alpha = 0.05$; data were log (x + 1) transformed).

Time (h)	df	F	*P*	Scheffé Comparison	Grand Mean	MSD
1	1, 18	192.47	0.0001	Ambient < cool	0.20	0.03
2	1, 18	65.34	0.0001	Ambient < cool	0.37	0.04
4	1, 18	6.85	0.0174	Ambient < cool	0.47	0.05
5	1, 18	0.31	0.5838	Nonsignificant	0.54	0.05
6	1, 18	3.18	0.0914	Nonsignificant	0.60	0.05
8	1, 18	1.89	0.1865	Nonsignificant	0.68	0.04
10	1, 18	0.35	0.5636	Nonsignificant	0.75	0.04
24	1, 18	6.91	0.0170	Ambient > cool	0.93	0.04

TABLE 5(b).

Simple comparison of differences in tapwater evaporation (%) over time for each level of treatment ($\alpha = 0.05$; data were log (x + 1) transformed).

Treatment	df	F	*P*	Scheffé Comparison	Grand Mean	MSD
Ambient	7, 63	1234.46	0.0001	1h < 2h < 4h < 5h ≡ 6h < 8h < 10h < 24h	0.77	0.07
Cool	7, 63	1452	0.0001	1h < 2h < 4h < 5h < 6h < 8h < 10h < 24h	0.84	0.05

comm.). What probably occurred in this study was initial excretion of body fluids into the mantle cavity where necessary to maintain the gill tissues in a liquid environment, followed by a loss of body fluid and water retained within the mantle cavity once morbidity progressed to the extent that muscle tone was reduced and active water retention ceased. This would explain why lost fluid had a bluish hue consistent with the oxyhemocyanin respiratory pigment in abalone blood (Barnes 1986) and why I observed episodic losses from some abalone into their containers during weighing.

Evaporative losses simulated using tapwater were small throughout the experimental period and between treatments. In contrast, losses of abalone fluid were greater than the tapwater losses and varied with each treatment temperature regime. Losses of around 4% or more of the fluid from abalone subjected to ambient temperatures warrant further consideration. I would have expected evaporation of this apparently higher viscosity fluid comprising seawater, blood, and mucous secretions to be less than for water. Nonetheless, evaporative loss was accounted for in the estimates and does not affect the conclusions from this study.

Results from this study have important implications for assessment and management of the Victorian abalone fishery. Post-harvest weight losses of 10–20% equate to the removal of 6–12% (about 350,000 to 700,000 average sized abalone) more than expected from the current TACs. Catches used to model the fishery will need to be adjusted to account for these quantities. Because exploitation of weight loss before notification is a recent phenomenon in the history of the fishery, inclusion of the additional quantities in catch inputs will produce model outputs showing a higher risk of decline in population projections. If these risks are deemed to be unacceptable, then management decisions to reduce the TACs may follow that will cost the Victorian abalone catching sector several million dollars in income.

Greatest losses occurred under conditions similar to those most commercially harvested abalone are exposed to during fishing, transportation, and storage before processing. Although losses are inevitable aboard diver vessels while at sea (typically for periods of 4–6 h), these can be offset by careful packing of bins, shielding from the weather, and periodically irrigating or deluging the bins with seawater. Losses estimated during the first experiment were determined after the abalone had been similarly exposed aboard a research boat. Clearly, there is an incentive for abalone processors to become adept at maximizing weight losses before notification for decrementing quota allocations. From a management perspective, it is important to consider whether these practices are desirable and equitable.

Processors exporting live abalone are unable to take advantage of the regulations and must pay more per abalone. This may be offset by higher than normal wholesale prices for live product. Commercial abalone divers harvesting abalone destined for canning are at a financial disadvantage from the additional effort expended to attain quotas, unless they are compensated by higher than normal beach prices. However, the high rate of vertical integration within the industry, where those in the catching sector are also involved in the processing sector, ensures this practice is at least tolerated by most divers. It seems that industry concerns expressed a decade ago have given way to acceptance of overnight

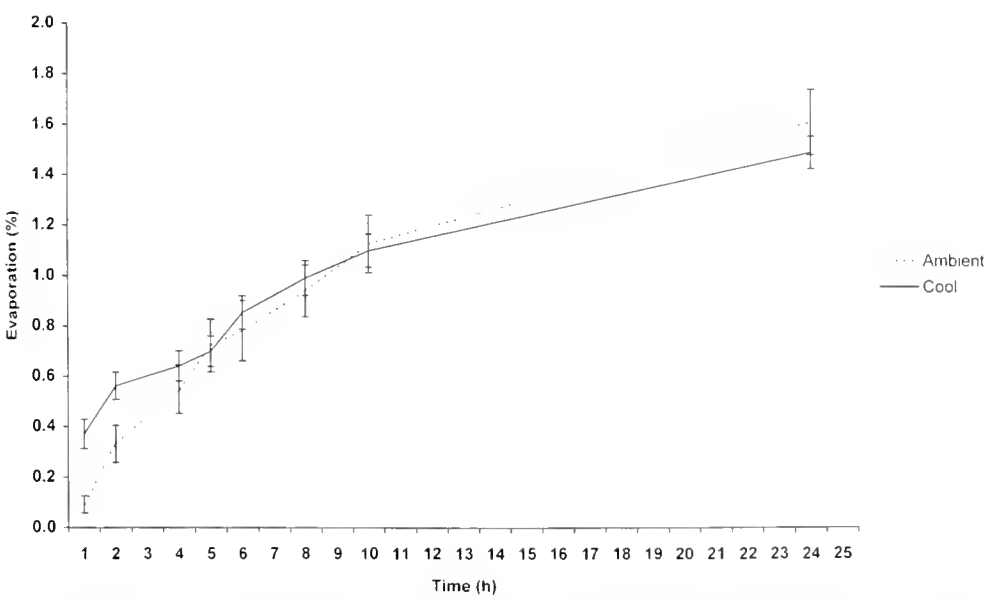

Figure 4. Mean cumulative evaporation of tapwater over time in ambient and cool storage environments (error bars are standard deviations).

drainage as standard industry practice. That most of the weight loss in the second experiment occurred during the first 8–10 h may partly explain why processors do not exploit the full 24-h post-landing period available, preferring, instead, only to allow catches to drain overnight. Alternative explanations include greater risk of spoilage if abalone remain unshucked for too long and cost efficiencies associated with the engagement of casual factory workers.

Evidently, the practice of maximizing weight loss through drainage does not seem to have a negative impact on the quality of canned or frozen abalone products produced in Victoria. Anecdotal information from persons with experience in the processing sector indicates that partial dehydration before canning may enhance the uptake of water into the meat. Some claim that increases of 20–30% are achievable, but this remains to be verified. However, Wells and Baldwin (1995) suggest the possibility that changes in metabolites produced during prolonged air exposure may affect the organoleptic qualities of abalone and cite several studies linking taste and meat quality to biochemical processes. This may be temperature dependent and less likely during refrigerated storage; however, it deserves attention.

In the future, it is probable we will see the introduction of official electronic beach weighing stations that will automatically decrement abalone quotas as catches are landed at major Victorian ports. The impetus for this will come as much from the need to reduce fishery management costs as to ensure compliance. Such practices are already part of the federally managed Southeastern Trawl Fishery in Australia and discussions have commenced regarding trial applications of electronic weighing for quota-managed fisheries under state jurisdiction. Until changes in the determination of official catch weights are implemented, post-harvest weight loss will need to be taken into account when setting annual catch quotas for the Victorian abalone fishery.

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This work was funded by the Abalone Assessment Subprogram conducted by the Marine and Freshwater Resources Institute on behalf of Fisheries Victoria. Cameron Dixon, David Forbes, Mark Ferrier, and Bruce Waters are thanked for diving assistance in collecting the abalone, and Cameron and David are also thanked for their assistance with weighing and recording. Sonia Talman provided considerable assistance with weighing and recording throughout the second experiment and made constructive comments on an early draft of the manuscript, for which I am grateful. In addition, Noel Coleman deserves thanks for his helpful suggestions on an earlier draft. Comments from an anonymous reviewer also improved the manuscript.

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TRIGGERS AND TARGETS: WHAT ARE WE AIMING FOR WITH ABALONE FISHERIES MODELS IN AUSTRALIA?

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ABSTRACT A variety of quantitative measures have been applied as reference points in the management of Australian abalone fisheries. In New South Wales changes in legal-sized and mature biomass will trigger management responses, in South Australia catch rates, size composition, and abundance indices provide target reference points, and in Tasmania, catch rates and catches have been used to provide triggers for management decisions. However, Victoria and Western Australia have yet to determine their reference points for abalone stock assessment. Victoria has been developing length-based fisheries models similar to those applied in New South Wales and is now confronted with the necessity of converting model outputs into decision-making criteria. A Victorian fishery management plan is also under development in which reference points will be specified within a risk-based matrix of catch control rules for quota adjustment. Recent biodiversity conservation legislation, compelling fisheries management agencies in Australia to demonstrate that export fisheries managed under their jurisdictions are ecologically sustainable, has increased the urgency to establish these reference points. The application of this legislation draws upon the "Principles and Criteria for Sustainable Fishing" of the Marine Stewardship Council in London. We considered a range of alternative measures for reference points that may be useful as triggers and targets applied in a stochastic framework for management decisions. Although not a modeling output, one of the more consistent nonbiological signals of localized depletion in the Victorian fishery relates to spatial allocation of effort at the scale of reef complexes. Reductions in annual effort applied to a particular reef system invariably precede significant decreases in abundance indices with typically large coefficients of variation and in catch rates characterized by hyperstability. Victorian abalone divers have high daily catch expectations and allocate their effort accordingly. Empirical reference points, such as effort allocations, provide utility for fishery management and can be readily assimilated and adopted by industry. Consequently, we conclude that in addition to modeling outputs, maintenance of reef-scale effort allocation and daily catch expectations should form part of a suite of fishery performance indicators and target criteria for the Victorian blacklip abalone fishery.

KEY WORDS: biodiversity, sustainability, reference points, abalone

INTRODUCTION

The Australian Commonwealth *Environment Protection and Biodiversity Act 1999*, effective July 2000, and proposed revisions to the *Wildlife Protection (Regulation of Exports and Imports) Act 1982*, mandate that Australian fisheries resources be fished in ways that can be demonstrated as ecologically sustainable. To provide guidance for assessing sustainability, the lead agency for these Acts, Environment Australia, has recently released "Guidelines for Assessing the Ecological Sustainability of Fisheries Management Regimes" (see www.erin.gov.au/marine/fisheries/assessment/guidelines.html). These guidelines are similar to the Marine Stewardship Council's "Principles and Criteria for Sustainable Fishing." There are two principles underpinned by a series of objectives and assessment criteria that will seriously challenge many conventional approaches to fisheries management, particularly with respect to ecosystem impacts. To satisfy Commonwealth Government requirements, each fishery must operate under a management regime that satisfies both principles.

The first principle relates to avoidance of overfishing and promotion of stock recovery of targeted fish populations. The second principle is concerned with protecting the supporting ecosystem by managing fishing operations so that impacts are minimized.

Of particular interest to abalone stock assessment biologists, are criteria under the first principle that there should be sound estimates of potential long-term productivity, target reference points for levels of fishing and limit reference points beyond which stocks should not be targeted. These are criteria that stock assessment groups should be able to address with existing data and assessment techniques. Criteria under the second principle relating to ecosystem sustainability and biodiversity are more esoteric. It will be difficult to demonstrate satisfaction of these criteria, given our relatively poor understanding of ecosystem processes in abalone-dominated communities and, more importantly, the lack of comprehensive and spatially representative data for these communities.

Although other abalone-producing Australian states have management plans, Victoria has only recently embarked upon the development of a plan that will meet the future needs of its abalone fishery. In a response to the new legislation, Fisheries Victoria has nominated abalone as the first fisheries resource for which they will pursue assessment by Environment Australia. Highest priority in this regard has been given to the determination of reference points for the fishery. Unlike many fin fisheries across the globe where target and limit reference points have become well-established in fisheries management (Caddy 1998), abalone fisheries have yet to embrace a standard suite of reference points. We have already modified and applied a length-based stock-reduction model with an assumed Beverton and Holt stock–recruitment relationship to assess the Victorian fishery, in a fashion similar to an approach used for the NSW fishery. There is an expectation that outputs from the model will form the basis for reference points in the management plan, as is the case in NSW. However, other states use different reference points, and an examination of the efficacy of a range of alternatives is warranted. We describe, and where possible, evaluate some of the biological and fishery reference points currently applied in Australia in an attempt to stimulate debate about the selection of attributes to trigger management decisions and target fishery performance.

MEASURING PERFORMANCE, SETTING TARGETS, AND RESPONDING TO TRIGGERS

Terminology describing reference points can seem somewhat confusing, given everyday usage and varying definitions of tech-

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nical terms. Caddy (1998) uses the terms target reference point (TRP) and limit reference point (LRP), respectively, to refer to target values that mostly relate to recovery of overexploited stocks and trigger values that represent thresholds for avoidance of overfishing. For the purpose of our discussion, we consider reference points to be values of biological and fishery attributes that can be used to gauge performance of the fishery. In this context, target reference points are desired values of selected performance indicators against which we can make assessments about the current status of an abalone stock; whereas, trigger points are threshold values below which there is an unacceptable risk of adverse consequences for the fishery. Invoking these triggers should initiate management intervention before the fishery enters an undesirable state. Beyond the trigger points, are limit points or values that when attained indicate collapse of the stock is imminent. The larger the margin between trigger and limit reference points, the more likely that the trigger will be activated before critically low levels of stock occur (Caddy 1998). In a stochastic framework, performance indicators should specify a reference point value, the relationship between an observed value and the reference point, and the confidence probability for acceptance that the relationship is true over a specific time period. Applying reference points in a risk analysis is preferable to relying on individual deterministic values, because it has the advantage of incorporating uncertainty in the decision-making process.

Catch Rate

Although much has been written about the lack of utility in catch per unit effort (CPUE) as it is currently reported by abalone divers in most parts of the world, some studies support a contrary view (Worthington et al. 1998). It is also generally acknowledged that catch rates play some role at a fine scale in determining whether a commercial abalone diver will persist in fishing a specific population. Stock assessment teams inevitably resort to commercial catch and effort statistics and trends in CPUE when alternative data about the fishery are scant or inconsistently collected. This is a reflection of the power that comes from having large amounts of data over a time scale consistent with the history of the fishery and generally at a high temporal resolution. CPUE is used as a reference point in the South Australian abalone management plan (Zacharin 1997) and, until recently, was also part of the Tasmanian plan (Anonymous 1997). The South Australian plan specified that for blacklip abalone, the reference point would be triggered if a change in catch rate within major fishing blocks of greater than 15% between years or greater than a 25% change over a period of 5 consecutive y was observed. In Tasmania, two catch rate trigger points were applied (Officer 1999). The Tasmanian coast is divided into eight regions further subdivided into 51 statistical abalone catch and effort reporting blocks. One of the reference points would be triggered if annual CPUE at the state level from diver returns fell below 95% of the CPUE for the reference year (1993, 1994, or 1995) with the lowest catch rate. The other reference point would be triggered if annual CPUE from diver returns for any region or block fell below 75% of the CPUE for the reference year (1993, 1994, or 1995) with the lowest catch rate for that region or block. Officer (1999) concluded that these reference points were inadequate to indicate whether or not recent catches were sustainable. The 1997 to 1998 fishery assessment showed that, at the higher spatial resolution, only one statistical block was close to being triggered and that, on a statewide scale, average catch rates had increased every year since 1990. However, catches in some blocks had increased to unprecendented levels (in some instances, doubling) so that past performance could not be used to gauge sustainability. Concerns were held that CPUE may not reflect changing abundance and that a better indicator of biomass change was required.

In Victoria, the abalone fishery is subdivided into three relatively large management zones, each spanning several hundred kilometers of coastline (Fig. 1). However, catch and effort data are reported for area codes of tens of kilometers and reef codes that generally represent headlands or reef complexes. Average catch rates for each zone have mostly increased during the past 20 y for which detailed records are available. Before we can interpret this as increased abundance, we need to eliminate the possibilities that such trends are consequences of increased diver efficiency, changes in commercial divers' interpretations of effort, or access to new fishing grounds.

During the history of the fishery, there have been a number of changes in the composition of the Victorian commercial abalone diver population and divers' incentives to fish. License transferability was introduced during 1984 on a two for one basis followed by quotas during 1988. Between 1984 and 1988, beach prices increased threefold, from about $AUD 5–15 per kg, pushing up the price of licences. Divers who bought into the fishery frequently took out large mortgages to pay for consolidated licenses, adding increased incentive to maximize the return for each unit of effort. Further changes occurred as access license owners "leased" their licenses to contract divers, now formally recognized under the Victorian Fisheries Act (1995) as nominated divers. These divers are generally younger and fitter than the "retiring" access license owners and are paid about 10% of the abalone beach price. Discussions with experienced divers suggest that there has been an increase in catching efficiency during recent years. In addition, we have repeatedly implored divers to record only bottom time as effort rather than time spent on the water. Compliance with this request would tend to decrease the effort reported for each unit of catch, although the extent to which this has affected CPUE is unclear. More revealing is the spatial disaggregation of effort into groups of reefs arbitrarily categorized by their long-term average CPUE. This clearly shows a trend toward effort being concentrated on those reefs that provide the highest catch rates (Fig. 2).

Discussions with divers confirm that before quota introduction, divers would often fish low-catch-rate reefs. Once quotas were introduced, there was no longer an incentive to fish the low-catch rate reefs, because quotas could be readily obtained from a smaller number of reefs where abalone were large and abundant. Indeed, we have clear evidence of the hyperstability of CPUE on a reef that recently collapsed in the Eastern Zone of the Victorian fishery (Fig. 3). The Skerries is a seal colony in a relatively remote and ostensibly pristine location. However, difficult but nonetheless available access via 70 kilometers of rough road allows abalone thieves to fish this location. Whether illegal fishing is to blame is unknown; however, what is clear is that declines in prerecruit abundance were followed by declines in the abundance of legal-size abalone at our fixed monitoring site (Fig. 4) and a concomitant decrease in effort (Fig. 3). In contrast, CPUE increased during many of the final days of effort before licensed divers decided harvesting abalone from this location was no longer worthwhile (Fig. 3). Big Rame Head, another important reef approximately 3.5

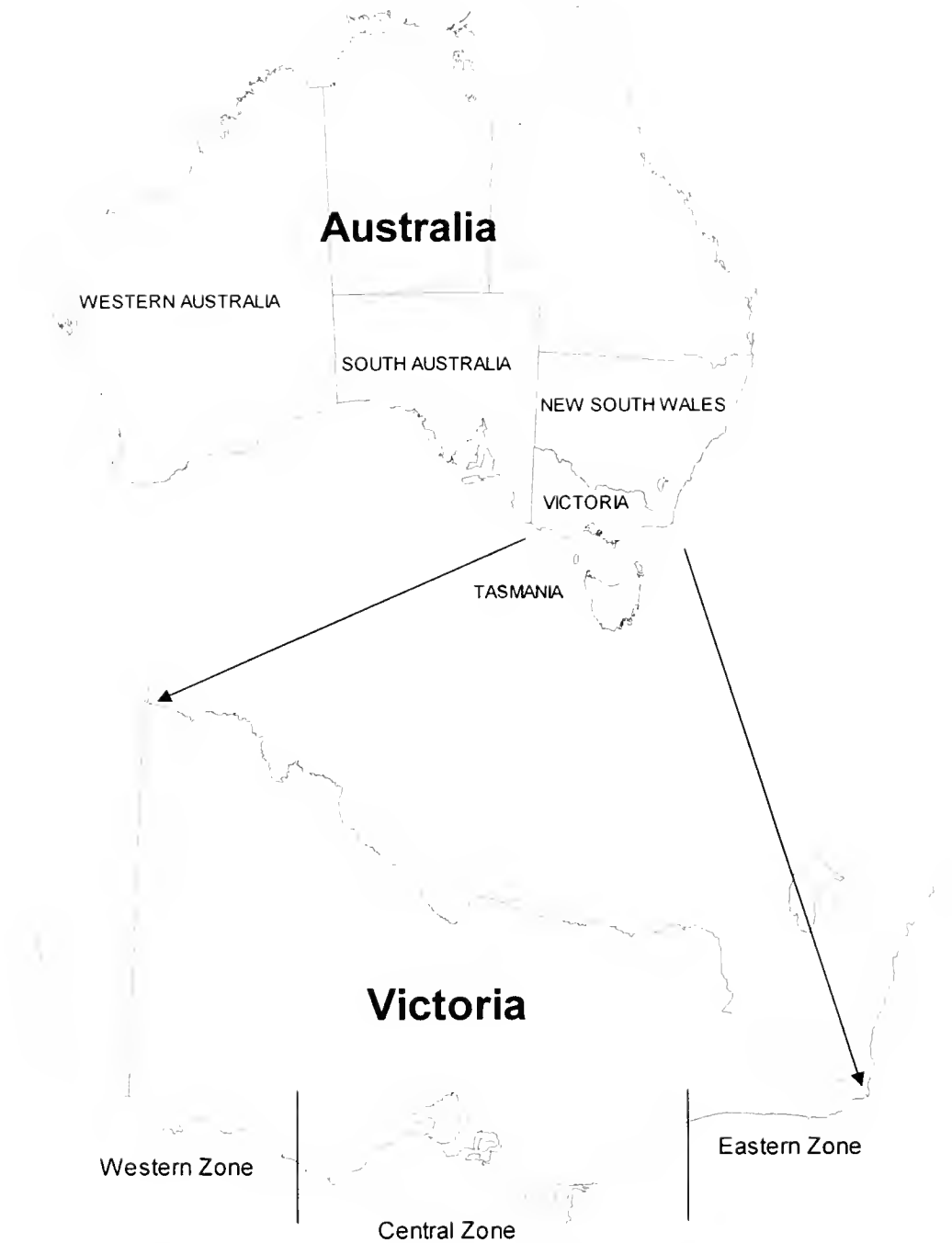

Figure 1. Coastline map of Australian abalone-producing states, with inset depicting management zone boundaries of the Victorian fishery.

km to the west of The Skerries, has contributed up to 8% of the Eastern Zone catch in previous years but is showing similar catch and effort trends to The Skerries (Fig. 5). However, declines in abundance were only recorded at the three of our five fixed monitoring sites closest to shore access and were not as substantial as declines at The Skerries.

Relative Abundance

Largely as a consequence of problems in applying conventional models to abalone fisheries (Breen 1992) and the failure of CPUE as an estimate of abundance (McShane 1992), the last decade has seen the introduction of independent dive surveys to estimate abundance of Australian abalone. It was anticipated at the outset that these surveys would provide a time series of relative abundance that would reflect changes in commercially fished abalone populations that could not be detected using catch and effort statistics (McShane 1994). The assumption was made that catch quotas would be adjusted in response to trends in abundance; however, until recently there was no explicit adoption of such adaptive management arrangements by any of the State fisheries authorities

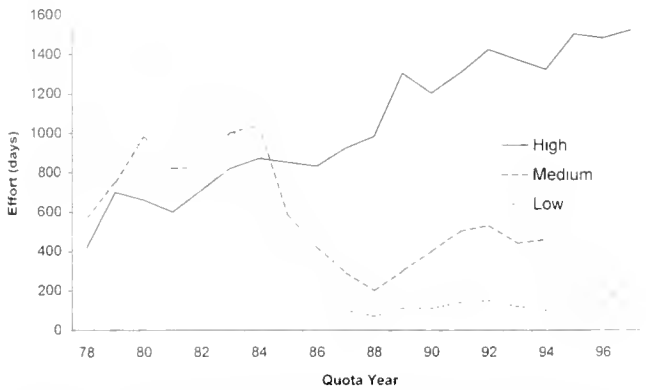

Figure 2. Annual effort (days) from 1978–1998 for reefs from the Central Zone of the Victorian abalone fishery with high (>61 kg/h), medium (55–61 kg/h) and low (< 55 kg/h) average catch per unit effort (CPUE).

responsible for abalone management. Indeed, abundance estimates on their own show sufficient spatial variability that the power to detect change is often low at the levels of replication allowed by available resources (Gorfine et al. 1998). Furthermore, lack of temporal resolution and infancy of these programs has made the detection of statistical trends in these data problematic. That these programs were initiated late in the development of the fishery, well after the initially rapid reduction in prefished biomass (Gorfine & Walker 1996), has meant that there is often little contrast in the temporal effect once residual variation has been partitioned from the data. Nonetheless, South Australia includes indices of abundance from independent dive surveys as one of the reference points in its abalone fishery management plan (Zacharin 1997). This reference point is triggered if a greater than 15% change is detected in the abundance of abalone above the legal minimum size, and abundance of prerecruits between consecutive years.

Our research on independent survey methods and experience in their application for large-scale monitoring during the past decade clearly demonstrates that effect sizes of 15% are unlikely to be detected given the large coefficients of variation (CV) for abundance estimates (Hart et al. 1997). CVs from unstandardized abundance data differ substantially among size classes. Our survey data show those abalone within 10 mm above and below the legal minimum length (LML) generally have CVs less than 30%, whereas the more cryptic smaller size-classes and sparsely distributed larger classes have CVs that make the detection of changes in abundance unlikely (Table 1). Although we have had sufficient statistical power with our methods to detect significant changes as small as 10% on some occasions (Gorfine et al. 1998), patterns in interannual variability fluctuate to the extent that a substantial increase between a pair of consecutive years may often reverse during the subsequent year. Management decisions, particularly about catch quotas, made in response to unstable patterns such as these will almost certainly create more problems than they solve.

In Victoria, we no longer use trends in fishery-independent abundance separately from catch data to assess stock status. Instead, we use the abundance estimates to fit fisheries models that incorporate commercial catch data to describe and forecast trends in relative biomass.

Egg Production

Shepherd and Baker (1998) suggest that egg production may be an acceptable substitute for biomass to predict risks of overfishing. Their eggs-per-recruit (EPR) analyses together with their review of EPR estimates from other studies indicates that the range 40–50% of virgin egg production is associated with sustained yields from medium to large abalone stocks and could be used as an appropriate threshold reference point. However, these values seem to have been derived from deterministic EPR models that take no account of the stochasticity in abalone growth described by Troynikov and Gorfine (1998). In addition, although it is reasonable to surmise that empirical evidence of sustained yield over a long period indicates a stable stock, it does not necessarily follow that the EPRs for a series of populations classified as either stable or collapsed can be collectively used to establish thresholds for EPR. In a review of EPR models, McShane (1995) describes their limitations in failing to account for variability in growth and natural mortality over small spatial scales and in assuming that fishing mortality and recruitment apply uniformly to the exploitable stock.

Differences of opinion exist as to the nature of stock-recruitment relations in abalone and whether or not conserving a particular level of egg production will ensure sustainability of an abalone fishery. Even if stochastic versions of EPR models were applied, the outputs would only allow selection of size limits, because these models provide no information about the effects of varying catch quotas.

Relative Biomass

The New South Wales stock assessment team use relative biomass outputs from their length-based fishery model as performance indicators linked to two trigger points that relate to the biological objectives of the Management Plan for the NSW Abalone Share Management Fishery (Worthington et al. 1999). These reference points are specified in a manner that accommodates the uncertainty associated with model outputs. The first reference point is a performance trigger that is invoked if there is more than a 50% chance that median estimates of legal and mature relative biomass for any region, or for the entire fishery, are less than 85% of the value for 1994. The second reference point is a forecast trigger invoked if model projections over the ensuing 5 y show a greater than 50% chance of the legal and mature relative biomass being less than 85% of estimates for 1994. During 1998, neither trigger was invoked, although at the regional scale, some B/B_0 values averaged only 2% greater than the 85% trigger point with likelihoods only 5–10% less than the 50% reference probability (Worthington et al. 1999). In the absence of target reference points implying the contrary, the premise underpinning this approach is that the state of stocks during the reference year (1994) was at an acceptable level.

In Victoria, we have recently applied our version of a length-based model (unpublished). Our model differs somewhat from the NSW model, because it incorporates a framework for dealing with variability in the distribution of growth within populations, and in its current form, it does not use effort. Like the NSW model, ours fits the model to several time series of abundances of different length classes obtained from fishery-independent dive surveys of commercially important populations (Fig. 1). Outputs from the Victorian model are essentially the same as those of the NSW model; however, in the absence of a management plan, there are no agreed reference points against which model outputs can be assessed.

What we have been able to do for the Victorian fishery is incorporate the model outputs into a risk analysis where future

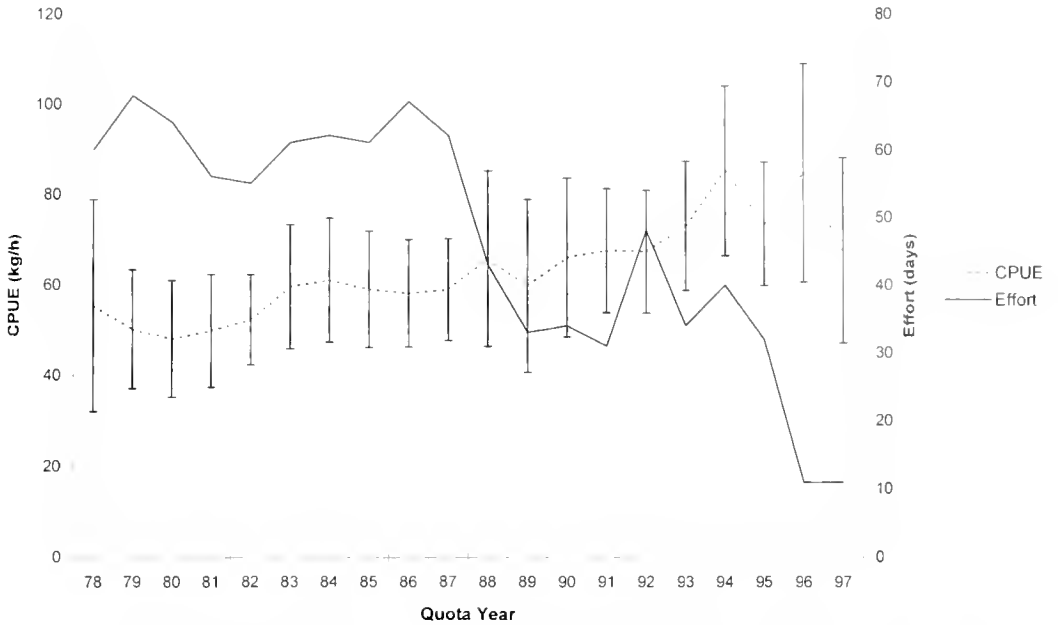

Figure 3. Catch per unit effort (mean kgh^{-1} ± SD) and annual effort (days) from 1978–1998 at The Skerries in the Eastern Zone of the Victorian abalone fishery.

biomass trajectories are predicted for a given harvest strategy (proportion of current TAC) at a selected level of risk. Figure 6 shows relative biomass projections up to 25 y for different proportions of the current TAC at three levels of risk (10, 20, and 30%) of being below the values of B_t/B_0 for each curve. Sensitivity of these projections to three combinations of natural mortality and unaccounted catch is shown. As would be anticipated, increasing the current TAC is likely to produce a decrease in biomass. In contrast, as the level of risk selected increases, the prognosis for sustaining current biomass improves. However, the projections are sensitive to levels of natural mortality and unaccounted catch. Because the model estimates its recruitment parameters internally, high mortalities throughout the life of the fishery will tend to make it more productive; whereas, increased mortalities in its more recent his-

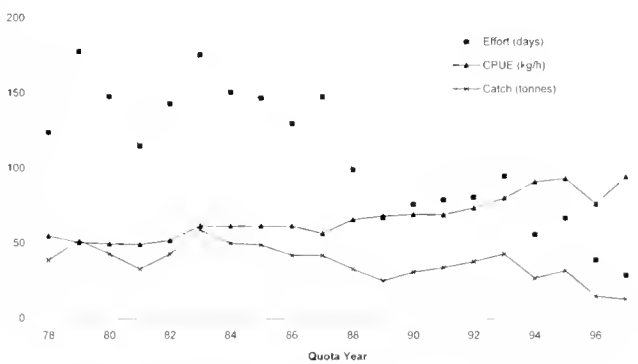

Figure 5. Annual catch (metric tonnes), catch per unit effort (kgh^{-1}) and effort (days) from 1978–1998 at Big Rame Head in the Eastern Zone of the Victorian abalone fishery.

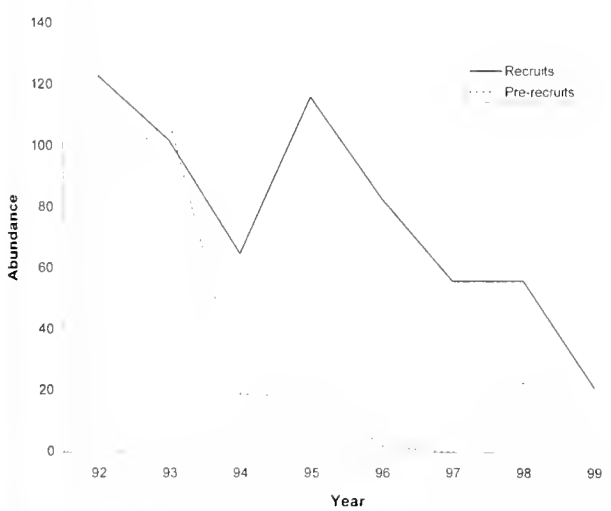

Figure 4. Relative abundance of legal size and prerecruit abalone from 1992–1999 at The Skerries in the Eastern Zone of the Victorian abalone fishery.

TABLE 1.

Ranges of coefficients of variation (SE/mean), during 1992–1998, for relative abundance estimates of five size-classes of blacklip abalone surveyed in the three management zones of the Victorian fishery.

Size-Class	Central Zone[a]	Eastern Zone	Western Zone
70–89 mm	1.10–2.09	—	—
80–99 mm	0.43–0.71	1.09–2.28	1.55–4.69
100–109 mm	0.14–0.23	0.50–0.74	0.48–0.80
110–119 mm	0.18–0.22	0.31–0.52	0.17–0.34
120–129 mm	0.82–0.89	0.18–0.27	0.09–0.12
130+ mm	—	0.31–0.58	0.46–0.84

[a] LML = 110 mm in most of this zone, as compared with 120 mm elsewhere.

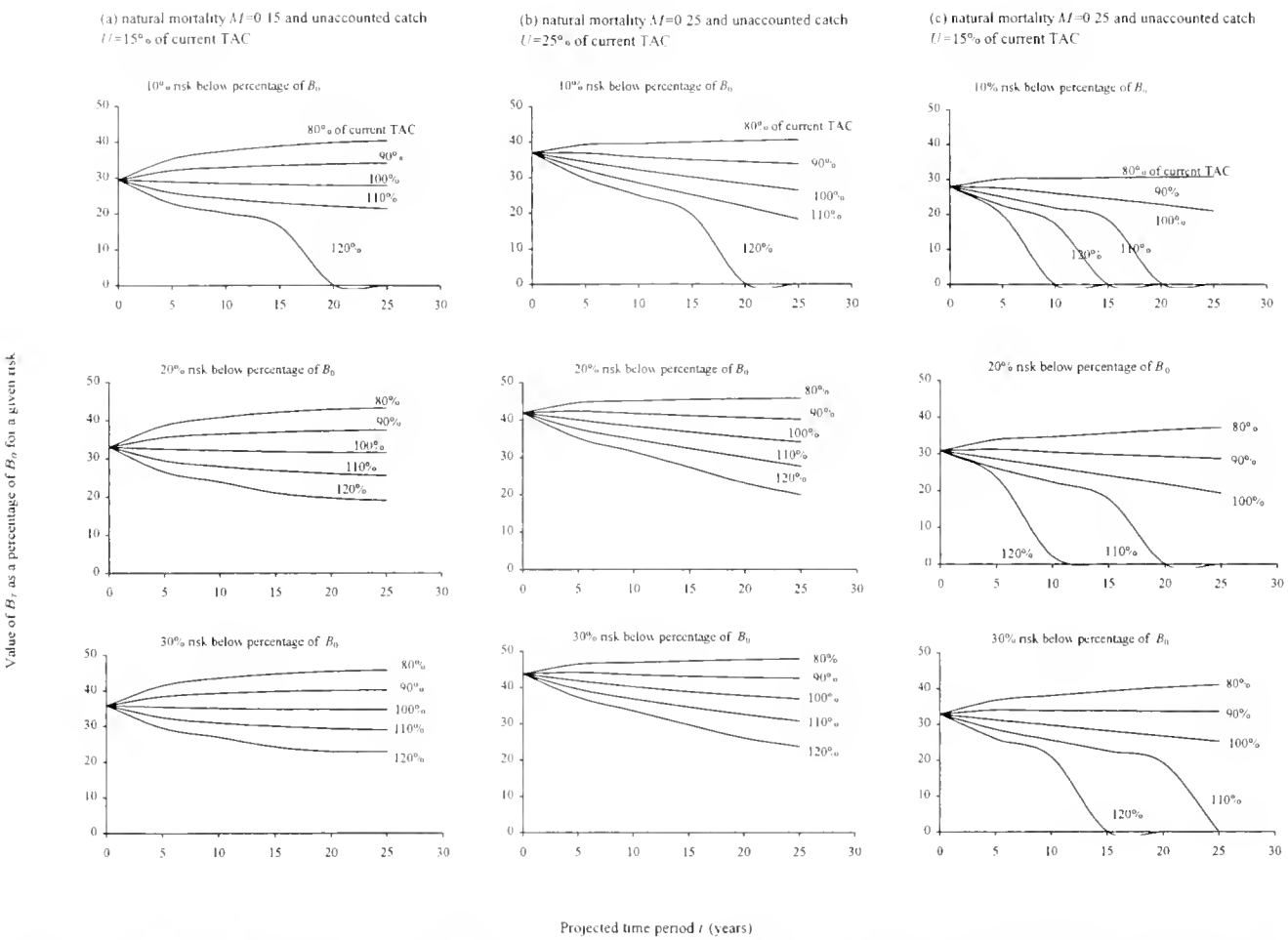

Figure 6. Relative biomass projections for the Eastern Zone for different harvest strategies at three risk levels showing sensitivity to three combinations of natural mortality and unaccounted catch.[B_t is biomass at projected time period t (years), B_0 is initial prefishing biomass, B_t/B_0 is expressed as proportion (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6), constant harvest strategy is expressed as a percentage of the current TAC (80, 90, 100, 110, and 120%), risk is expressed as a percentage probability (10, 20, and 30%) that the true value is below the estimated values for B_t/B_0, and the projected time period is t years beyond 1998 (t = 0, 5, 15, and 25 years). U is the unaccounted catch as percentage of current TAC and, M is the rate of natural mortality].

tory will tend to have the opposite effect. Such issues as when illegal fishing became a problem and whether much catch went unreported in the early years of the fishery can have profound effects on outputs from the model.

Notwithstanding the preliminary nature of these results, depletion curves from model outputs show that current biomass in the Victorian fishery is about 30% or more of the prefished biomass and 97% of the 1988 biomass (Fig. 7). Although stocks generally seem to remain in slow decline since 1988, and for future projections, most of the depletion of prefished biomass occurred during the first 5 y of the fishery, well before quota introduction.

Commercial Effort

Victorian abalone divers have high daily catch expectations and allocate their effort accordingly. Detailed observations aboard commercial abalone vessels show that these expectations are typically about 500 kg for 5 h spent at sea, but may be as high as 1,000 kg (Gorfine & Dixon, this publication). Where divers anticipate lower catches, this almost invariably reflects the balance of unharvested quota on their last day of fishing for the year. These high catches cannot be obtained unless divers select reefs where experience has demonstrated that aggregations of abalone are numerous and dense. Divers will generally also have some perception of how well these populations recover after fishing and when they were last visited. Experienced divers will occasionally have to perform several test dives to locate good patches of abalone if recent intensive fishing has occurred, and inexperienced divers may make many such dives if their local knowledge is limited.

Insights from on-board observations may lead us to develop alternative reference points based on temporal and spatial allocation of effort. The basis for this assertion is that divers respond to change in stock availability that is below the detection limits of analyses of reported catch-effort and fishery-independent abundance indices. However, we must first account for variables that tend to confound this interpretation of effort, such as weather, diver experience, and market requirements. One possible empirical reference point could include reef-scale effort allocation, with a trigger point set at numbers of days per reef falling below some margin of the average number of days per annum since quota introduction. Another reference point could be related to daily catch expectation with a trigger that is activated if average daily catches fall below a specified margin of an historical average.

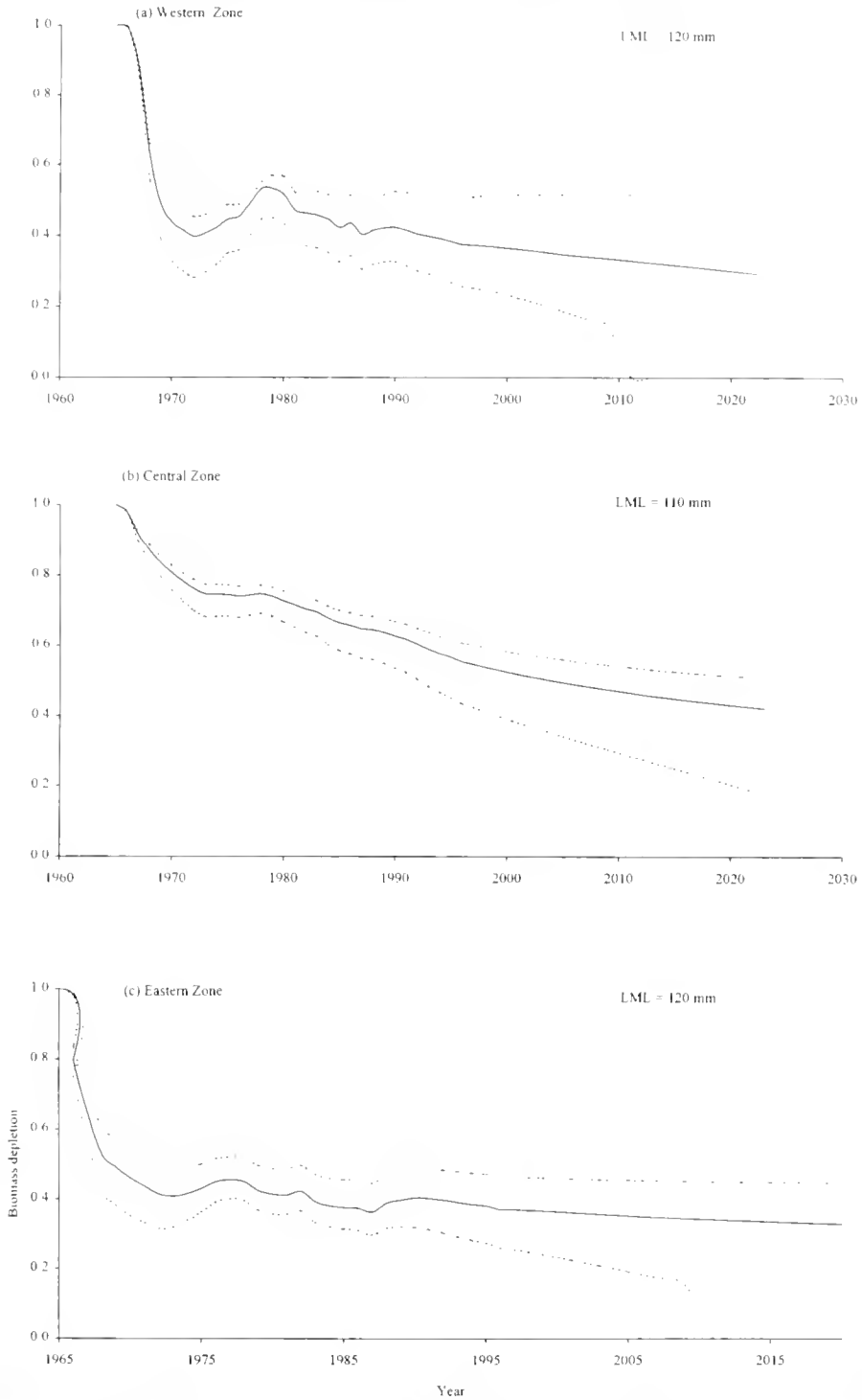

Figure 7. Biomass depletion (with 95% probability interval) for legal-size abalone (projections with current TAC and assumed values of unaccounted catch) in the (a) Western Zone, (b) Central Zone, and (c) Eastern Zone of the Victorian abalone fishery.

As well as changes in CPUE, the Tasmanian management plan specified percentage changes in catch for reporting regions relative to 1995 as trigger points. However, Officer (1999) reports that spurious activation of these triggers occurred in instances where regional catches were exceptionally high during 1995. He also points out that activation of these nonbiological triggers provides no indication that increased catches are unsustainable.

DISCUSSION

Catch per unit effort seems to have little to offer as a reference point for abalone fisheries. Its insensitivity to changing abundance and its sensitivity to changes in the composition and behavior of the diver population ensure that it reflects the state of the predator rather than its prey.

Shepherd and Baker's (1998) %EPR as a reference point provides no information about sustainable catch quotas. However, their conclusion that sustainability for small abalone populations may require the conservation of reproductive capacity at levels nearing 100% of the prefished stocks is consistent with Prince and Guzmán del Próo's (1993) stock reduction analysis of several zones in the Mexican fishery. This Mexican fishery analysis showed the zones to have poor productivity with little surplus yield to support sustained fishing. In another study of the Mexican fishery, Ramade-Villanueva et al. (1998) found that catch quotas based on conserving 75% of the current fishable biomass were too high to prevent recruitment overfishing.

The direct application of current measures of relative abundance as biological reference points is unlikely to be effective for managing abalone fisheries. Incorporation of independent observations of relative abundance into the model fitting process provides more information about trends in a stock than can be gained by the use of abundance indices on their own. However, we must continue to refine our survey methods to reduce the inherent variability in abundance data, because catches must cause large changes in relative abundance for depletion models to function effectively (Walters 1998).

Relative biomass seems to be the most efficacious among performance indicators linked to abalone fisheries models. However, choice of reference year for the denominator of the biomass ratio will depend on some independent information about the fishery during the year selected. For the NSW fishery, 1994 was chosen, because it was the year during which independent surveys of abalone abundance commenced (Worthington et al. 1999). In Victoria, a similar approach could be used by selecting 1992, when we commenced our current series of relative abundance surveys; however, it may also be informative to specify 1988, the year of quota introduction, as the reference year. This is because quotas were introduced at a time when yields from the fishery had stabilized, and managers and industry aspired to maintain stocks at current levels rather than to allow catches to increase and risk overfishing the resource.

In many fisheries, biomass reference points are set at specific values relative to an estimated prefishing or virgin biomass value B_0. For example, $B_t/B_0 = 30\%$ has often been used as a reference point for scale fisheries, particularly in the northern hemisphere (Quinn & Deriso 1999). However, there is no evidence that this or similar values have any relevance to such invertebrate fisheries as abalone. One of the main reasons for selecting a proportion of B_0 as a threshold is the assumption that decreases in biomass are reversible (Perry et al. 1999) and that stocks can be restored to former abundance (Beverton 1990). Although this may be true to some extent for abalone within a metapopulation, it is possible that at low abundances, depensatory effects may lead to irreversible recruitment failure for high levels of exploitation at this scale (Shepherd & Partington 1995). Under these circumstances, it is likely that serial depletion of metapopulations will mitigate against the rebuilding of stocks at the fishery scale.

An important consideration in the selection of biomass reference points is the level of uncertainty in the biomass estimates. Estimates for those years where data exist to fit the model (in our case, the 1990s) will provide less uncertainty than for past years, when reliable fitting data were unavailable. Similarly, the further into the future that biomass estimates are projected, the greater the uncertainty. Although the examples in this study show projected biomass estimates over 25 y for illustration, in actual application, a projection period of, say, 5 y would be more reasonable.

Although intuitively appealing, relative biomass projections can be problematic. As with all models, sensitivity to parameter values and the capacity to estimate these values empirically are issues that must be resolved. It is almost trivially obvious that a model dependent on catch loses its reliability when a large proportion of the catch remains unaccounted. However, this is one of the major problems facing all Australian abalone fisheries. Factoring notional unaccounted catches into a model can be counterintuitive, because, as described above, historically large catches tend to provide less conservative projections. Consequently, we are planning to direct future research efforts to developing robust methods for estimating illegal catches.

Our model outputs highlight the important drawback with constant harvest strategies that, when biomass is declining, the TAC represents an ever-increasing proportion of the available stock (Perry et al. 1999). In the absence of timely management intervention, it is reasonable to expect declines in biomass to accelerate under quota management until some threshold is passed, and the stock suddenly collapses. The modeling also shows that the introduction of quotas was not responsible for arresting declining biomass. The slowing of biomass depletion resulted from diver attrition during the 1960s and 1970s when, due to low beach prices and the introduction of fees, many Victorian divers decided there were insufficient incentives to continue fishing. A constant rate of fishing mortality may seem a better option than quotas, and this is commonly used in scale fisheries. McAllister and Kirkwoood (1998) found that effort control management options outperformed catch control options in simulations by providing 40% more catch for the same risk of stock depletion. However, this approach increases the complexity and intensity of management decisions (Perry et al. 1999)

Although relative biomass seems to offer the most promise for developing abalone reference points, in the short term there must be alternative pathways when the quantity and quality of available data inputs and parameter estimates are poor or model assumptions are substantially violated (Walters 1998). Reference points must embrace not only model outputs but also other factors that are not modeled. For example, until we have sufficient data to disaggregate our model spatially, we require reference points suitable for addressing fine-scale changes in stocks at the scale of bays and headlands. Reference points also must be sufficiently diverse to provide empirical alternatives as reality checks on models.

The use of effort as an indicator of stock status may be of benefit under such circumstances; however, spatial and temporal shifts in effort must be treated cautiously so that reasons unrelated to the state of the resource can be eliminated. The Tasmanian observations on the application of such nonbiological indicators as patterns in catch underscore the importance of careful selection of temporal baselines and the need for analysis on the finest spatial scale practicable. It may be possible, however, to use average values of daily catch expectation and aggregate days of effort per reef as performance triggers that initiate management action when some predetermined threshold is passed. Indeed, Walters (1998) makes the point that there is potential for novel indicators of overexploitation to be developed based on the detailed information available in catch statistics. We believe the spatial resolution in catch and effort data has yet to be used to its full potential in managing abalone fisheries.

The adoption of multispecies approaches, focusing on so-called

keystone species in addition to the target species is a noble and possibly essential goal. However, our current survey techniques are already costly and, consequently, can only accommodate abundance estimates of relatively few organisms. It is unclear how we can attempt to model abalone ecosystems until we have a better understanding of density dependence, interspecific competition, and trophic interactions.

Whatever reference points are adopted for the Victorian fishery and incorporated into its management plan, involvement of stakeholders will be a critical part of the reference point selection process to ensure that triggers and targets are meaningful and of practical value. An inclusive process of involving stakeholders in the prenegotiation of reference points is also required to ensure consensus about management prescriptions (Caddy 1998). Without ownership of the management process by stakeholders, compliance and reporting problems will prevail under adverse circumstances. Access license owners will be wary of providing any information that they perceive may decrease the value of their entitlement, both in direct monetary terms and as a mortgageable asset. Contract divers (and processors) make their money on the quantity of quota they can negotiate to catch on behalf of license owners. Understandably, their focus is on the short- rather than the long-term situation; hence, a rush on declining stocks to get the most out while still able would be inevitable.

The establishment of a suite of empirical and model-based reference points within an agreed decision-making framework in a management plan underpinned by comprehensive catch and effort reporting and broad-scale fishery and habitat monitoring should ensure that the Victorian abalone fishery satisfies the ecological sustainability requirements of Commonwealth legislation. As a secondary benefit, this may provide the Victorian abalone industry with a competitive edge in marketing its products as "environmentally friendly."

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TOWARD ASSESSING THE SOUTH AFRICAN ABALONE *HALIOTIS MIDAE* STOCK USING AN AGE-STRUCTURED PRODUCTION MODEL

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ABSTRACT A deterministic age-structured model is developed for use in estimating resource dynamics parameters and projecting biomass trends for the South African abalone *Haliotis midae*. This constitutes the first quantitative approach applied to the management of this commercially valuable resource. The model focuses on one of seven commercial fishing zones, zone C, because particular concerns have been expressed about the status of the resource in this region, *inter alia* because of the considerable catches taken by the illegal fishing sector. The modeling approach is summarized, and some preliminary results from a single application of the model are given. Model parameters including the pre-exploitation spawning biomass B_0^y, the present annual poaching take CP_{max} and the natural mortality M, are estimated by fitting the model to CPUE data for the period 1980 to 1998, Fishery-Independent Abalone Survey (FIAS) abundance data as well as several years of catch-at-age data for each of various components of the fishery. The CPUE data are standardized using a general linear model, and zone C is split into two "linked" areas, a "poached" and "nonpoached" subarea with common resource dynamics parameters but separate fishing histories. This was necessary because of substantial differences in both the relative abundance trends and catch-at-age information for these two areas. Model results suggest that the resource in the "poached" and "nonpoached" areas of zone C is presently at ca. 1% and 85%, respectively of its pre-exploitation level (B_0^y = 3,360 and 1,580 tons, respectively). The model-estimated annual poaching catch for zone C from 1995 to 1998 (240 tons) exceeds the annual commercial catch in zone C over this period. The model indicates that the maximum sustainable yield (MSY) is reduced by more than one-third if catches include the proportion of animals substantially smaller than the minimum legal-size limit thought to be taken by poachers. Model predictions should be interpreted with caution, because more work needs to be done to improve and evaluate the confidence that can be attached to model predictions and to refine the structure and assumptions of the model. A number of associated suggestions are presented.

KEY WORDS: *Haliotis midae*, abalone stock assessment, age-structured production model

INTRODUCTION

The South African abalone fishery is one of the oldest commercial abalone fisheries in the world, with commercial catch data having been recorded since 1953. The fishery is reliant on a single species, *Haliotis midae*, which is restricted to shallow habitats in beds of kelp, *Ecklonia maxima* (Tarr 1993). Aspects of the biology, including growth, reproduction, and movement, have been well studied and Tarr (1993) summarizes a broad overview of the biology of *H. midae*. A description of the fishery is presented in Tarr (1992) and Tarr (in press). Briefly, the commercially fished area has been divided into seven fishing zones, with total allowable catches (TACs) set separately for each zone over the last 13 y. The main fishing areas are zones A–D (Fig. 1). Uncertainty and concern have been expressed regarding the status of the abalone resource, which is subject to particularly high levels of illegal fishing and an ever-increasing number of recreational divers (Tarr in press). The magnitude of the catch taken by the illegal sector is unknown, and it is difficult to estimate in the field because of the evasive behavior of the illegal fishers (poachers). Spokespersons for the illegal sector have claimed that their catch is approximately half that of the legal commercial fishery in the area. This study represents a first attempt to estimate the illegal catch in zone C by using an age-structured model that simulates plausible abalone resource dynamics. This model provides a basis used at present to develop management advice for this resource by projecting abundance trends under alternative future catch levels. The model is applied in the first instance to zone C because of the severe stock depletion thought to have occurred in this zone and, hence, the urgent need for a more rigorous quantitative stock assessment. Furthermore, the data for zone C exhibit more contrast than the data for the other zones, so that it seems sensible to apply the model first to this area, because the contrast suggests better potential for precise parameter estimation. Such estimated parameter values might be input to applications of the model to other zones where data contrast is insufficient to allow their independent estimation.

Several different stock assessment approaches have been used in abalone fishery management and a review of these is given in Breen (1992). One of the greatest impediments in the application of stock assessment models to abalone fisheries has been that such models usually rely on catch per unit effort (CPUE) data as an index of abundance. Several authors have stressed that CPUE is not a reliable index in situations where the stock is highly aggregated, as occurs for example off southeastern Australia (Breen 1992, Keesing & Baker 1998). We argue that CPUE data have utility in the South African context, especially when used in conjunction with a fishery-independent index. A second impediment to applying standard stock assessment models to abalone populations has been the lack of adequate data on aging. Tarr (1995) used a tagging study to estimate growth rates of *Haliotis midae*. This, in turn, allowed the age structure of catches to be estimated, and consequently, we were able to construct the first fully age-structured population model to assess the status and productivity of the South African resource. This approach could be adapted for use in other areas of the world once aging techniques are refined. The other major requirement would be the availability of a reliable index of abundance for the stock in question. Moreover, the population dynamics model would have to take account of spatial variability in regions characterized by great spatial variation in demography.

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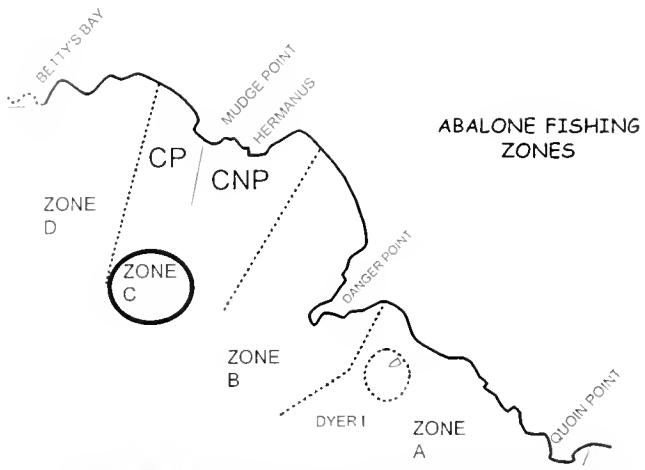

Figure 1. Map showing the four main commercial fishing zones defined for abalone *Haliotis midae* in South Africa. This study focuses on zone C, which has been split into a "poached" subarea (CP) and a "nonpoached" subarea (CNP).

DATA

Several historic changes in the commencement and closure dates for the commercial fishing season (Moloney 1999) necessitated all available data being reworked in terms of a standard fishing year y that is taken to run from October of year y-1 to September of year y. Furthermore, previous analyses (e.g. Plagányi & Butterworth 1999) showed that it was difficult to interpret the abalone dynamics in zone C without consideration that the western and eastern areas of this zone differed with respect to both density and size composition (Figs. 2, a,b). For assessment purposes, zone C has, therefore, been split into a "poached" subarea (CP) to the west (Hawston/Mudge Point areas) and a "nonpoached" subarea (CNP) to the east (Hermanus vicinity) (Fig. 1). Although some poaching is still thought to occur in the "nonpoached" subarea, this is thought to be considerably less than that in the "poached" subarea. The two areas are approximately equivalent in terms of available habitat for abalone, because the kelp bed area estimates for areas CP and CNP are 1.38 and 1.59 million square meters, respectively (Tarr 1993). In terms of the length of coastline, CP is approximately half of CNP's length.

Total allowable catches for the commercial fishery were set individually for each of seven fishing zones A to G from the 1986–1987 season onward (Tarr 1992). Moreover, data on the commercial catch of abalone in zone C are available for the period 1977 to 1985. However, before 1972 catch data are available only for all zones (A–G) combined, and, hence, zone C catch estimates for the period 1953 to 1976 are assumed to be a fixed proportion p_c (27%) of this total annual catch, where p_c is the average of zone C's proportional contribution to total catch over the period 1977 to 1981. The same approach is used to apportion the zone C catch estimates for the period 1953 to 1976 between the two subareas CP and CNP. The resultant time series of total commercial catches for zone C as a whole are listed in Table 1.

Table 1 also shows recreational catches. These are estimated from telephonic surveys, conducted since 1992, of selected recreational permit-holders. Recreational catches before the 1990s are thought (Tarr, pers. comm.) to have been negligible relative to the commercial catch, so that a low fixed amount of 2 MT/y was assumed in the model. In contrast to the commercial fishery, where

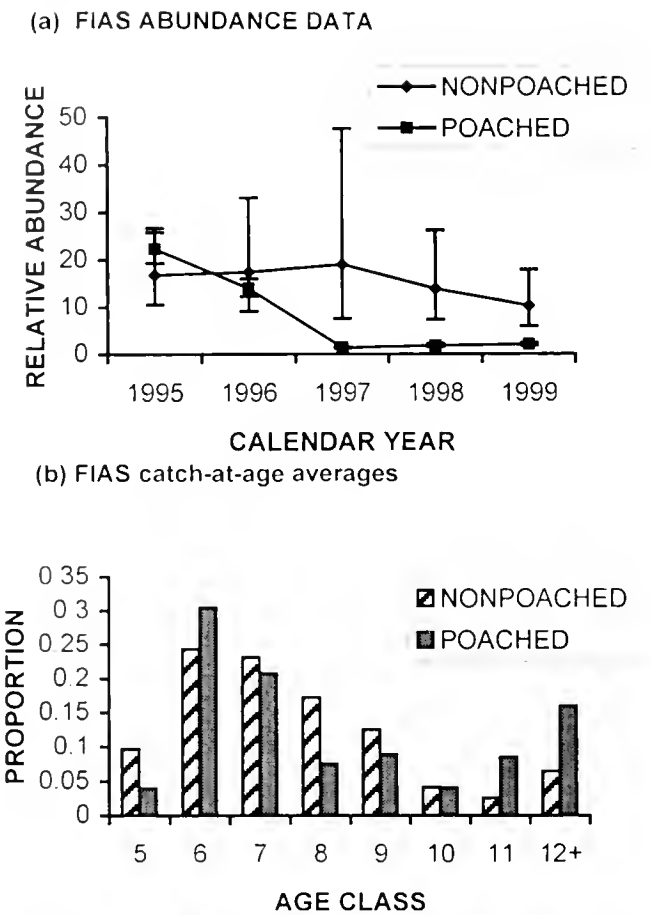

Figure 2. Comparisons between FIAS data obtained for the "poached" and "nonpoached" subareas of zone C. (a) shows the trend in the relative abundance index (average number $\bar{x}$ per 60 m^2) in each subarea. The error bars indicate the 95% confidence intervals calculated as $\bar{x} * e^{\pm 1.96 \cdot CV}$; i.e., assuming a log-normal error distribution. (b) compares the catch-at-age data averaged over the period 1995–1999. The catch-at-age data are derived from length distribution data (from A. McKenzie, MCM, RSA) that is cohort-sliced using a von Bertalanffy growth curve (Tarr 1995).

a law in force since 1966 prohibits commercial abalone fishing operations within 185 m of the high-water mark (Dichmont et al. 2000), the recreational fishery is essentially a shallow-water fishery with divers accessing the resource mostly from the shore. The relative proportions of the zone C recreational catch taken from the two subareas CP and CNP was, therefore, assumed to be proportional to the relative lengths of the coastline (CP:CNP = 1:2).

Total annual catch in each of the zone C subareas is calculated in the model as the sum of the commercial, recreational, and model-estimated poached catches. Analysis of the proportion of the total commercial catch caught during a season indicates that the majority of commercial catches are landed during the first two quarters of the fishing year (i.e., October to March) (Table 2). The fishery is, therefore, modeled as a pulse catch after the first quarter of the fishing year; i.e., at the start of the calendar year. Before 1985, an overall catch limit was allocated for abalone and recorded by calendar year (Dichmont et al. 2000) so that the proportion of the total catch taken in the last quarter of a calendar year was relatively minor (Table 2). Therefore, it may safely be assumed that no adjustment is necessary to equate catches recorded for a

TABLE 1.

Commercial catches (MT) from 1953 to present calculated for the "poached" and "nonpoached" subareas of Zone C (the total for Zone C is also shown).

	Commercial			Recreational
Fishing	CNP	CP	C total	C total
1953	51.4	162.7	214.1	2
1954	98.6	312.2	410.8	2
1955	36.0	114.1	150.1	2
1956	29.7	93.9	123.5	2
1957	39.6	125.5	165.1	2
1958	38.2	121.1	159.3	2
1959	33.2	105.3	138.5	2
1960	83.1	263.2	346.3	2
1961	98.1	310.5	408.6	2
1962	142.1	450.1	592.2	2
1963	114.4	362.3	476.7	2
1964	131.3	415.8	547.1	2
1965	189.7	600.8	790.6	2
1966	174.8	553.5	728.3	2
1967	141.3	447.4	588.6	2
1968	95.3	301.7	397.0	2
1969	75.6	239.4	315.0	2
1970	66.5	210.5	277.0	2
1971	46.3	146.5	192.8	2
1972	50.3	159.2	209.4	2
1973	51.1	161.9	213.0	2
1974	49.6	157.1	206.6	2
1975	51.6	163.4	215.0	2
1976	50.1	158.5	208.6	2
1977	42.0	133.0	175.0	2
1978	49.9	158.1	208.0	2
1979	55.7	176.3	232.0	2
1980	17.1	160.9	178.0	2
1981	38.4	140.4	178.9	2
1982	26.7	131.2	157.9	2
1983	36.8	105.2	142.0	2
1984	70.2	89.0	159.2	2
1985	38.8	101.1	139.9	2
1986	57.3	120.6	178.0	2
1987	29.7	126.4	156.1	2
1988	26.2	139.5	165.6	2
1989	27.8	135.6	163.4	2
1990	40.1	116.6	156.7	2
1991	41.0	119.0	160.0	2
1992	30.5	113.9	144.4	45
1993	31.9	105.2	137.1	84
1994	44.1	91.3	135.3	70
1995	39.1	84.7	123.7	83
1996	66.8	62.7	129.6	70
1997	36.9	16.1	53.0	91
1998	23.5	7.8	31.3	12

Fishing years are taken to run from October to September, so that 1998 (for example) refers to the period October 1997 to September 1998. The last column shows the estimated catch (MT) by recreational permit holders in Zone C (data from Moloney 1999), where catch biomass was calculated by multiplying numbers by 0.7 kg, the average mass of an abalone of the minimum legal size. Strictly this average mass is somewhat larger, and could be evaluated using the estimated recreational fishing selectivity function (see Table 7), but this complication has been ignored for this initial analysis.

calendar year before 1980 with the catch corresponding to a fishing year running from October to September.

The recreational fishing season during the early 1990s ran from November to July, but as a result of large increases in the recreational catch, the recreational fishing season was reduced from 9 to 6 mo (1 November–30 April) in 1996–1997 and then reduced further to 4 mo (12 December–13 April) as from 1997–1998 and restricted to weekend and public holidays only from the 1998–1999 season (A. MacKenzie pers. comm.). From a modeling perspective, the implications of the recreational fishing seasons are that no adjustments are necessary to equate recreational catch estimates with the catch corresponding to a fishing year as defined above. Moreover, because most recreational fishing is likely to take place during the austral summer months, it seems reasonable to model the recreational catch also as a pulse catch after the first quarter of the fishing year. The same approach is used to model the poaching catch, supported by the observation (A. MacKenzie, pers. comm.) of a slight decrease in poaching confiscations during the winter months.

An important additional data source for the abalone resource is the Fishery-Independent Abalone Surveys (FIAS) conducted since 1995 (Table 3). These surveys were designed to provide an index of relative abundance with a CV of some 25% (which is substantially more precise than that achieved in earlier surveys) (Dichmont et al. 2000). The surveys involve twenty equidistant transects of 30-m length that run perpendicular to the shore in each of zones A–D, F, and Dyer Island. Two divers count and measure abalone encountered within a 1-m swath on either side of the transect. Only animals larger than 100 mm shell length are recorded so as to reduce uncertainty in the estimates attributable to the nonemergent/cryptic behavior of juveniles. This corresponds to a minimum sampling age of approximately 5 y. Because the "poached" subarea CP includes only approximately one-third of zone C's coastline, 6 of the 20 FIAS transects are located within subarea CP versus the 14 transects located within subarea CNP. This obviously has some implications on the CVs attached to the "split" estimates, with CVs in subarea CP ranging between 38–67% versus 24–47% for CNP (Table 3).

Data Used in the Model-Fitting Process

The population model is fitted to standardized CPUE data available for the period 1980–1998 for each of the two subareas constituting zone C (Table 4) as well as to the available FIAS data for each of the two subareas (Table 3). The catch-at-age data used in the model-fitting process are given in Tables 5a–e and include all available data from the commercial, recreational, poaching, and fishery-independent sectors. The catch-at-age data are derived from length distribution data (from A. McKenzie, MCM, RSA) personal communication that are cohort-sliced using a von Bertalanffy growth curve (from Tarr 1995) that was fitted to tagging data. In several instances, age classes have been lumped together to reduce the number of categories containing a proportional abundance of less than 2% in any one year.

METHODS

Standardizing the CPUE Data

A general linear model (GLM) was used to standardize the commercial abalone CPUE time series of abalone for the influence of other factors on the CPUE apart from resource abundance. The

TABLE 2.

The proportion (by mass) of the total commercial catch from 1980 to present caught during a season as shown.

	Season				
Fishing yr	4 (Oct.–Dec.)	1 (Jan.–March)	2 (April–June)	3 (July–Sept.)	4 & 1
1980	0.00	0.55	0.19	0.26	0.55
1981	0.02	0.59	0.16	0.23	0.61
1982	0.11	0.51	0.19	0.19	0.62
1983	0.06	0.43	0.22	0.29	0.49
1984	0.00	0.55	0.23	0.22	0.55
1985	0.07	0.53	0.20	0.20	0.60
1986	0.48	0.27	0.22	0.03	0.74
1987	0.50	0.30	0.20	0.00	0.80
1988	0.48	0.29	0.22	0.01	0.77
1989	0.56	0.33	0.11	0.00	0.89
1990	0.45	0.54	0.01	0.00	0.99
1991	0.58	0.29	0.10	0.04	0.86
1992	0.56	0.35	0.01	0.08	0.91
1993	0.85	0.15	0.01	0.00	0.99
1994	0.78	0.19	0.02	0.02	0.97
1995	0.75	0.18	0.06	0.01	0.93
1996	0.14	0.72	0.10	0.04	0.86
1997	0.40	0.16	0.05	0.40	0.55
1998	0.02	0.59	0.27	0.12	0.61

Fishing years y are taken to run from October of year y-1 to September of year y.

explanatory variables included in the GLM were those selected by Moloney (1999). The same is true for the interaction terms in the model. The purpose of refitting a GLM to the abalone data was to update Moloney's results to allow for splitting zone C into two subareas (CP and CNP), and for the fact the "fishing year" has been changed from a calendar year to a year running from October through to September.

The GLM model applied is given by:

$$\ln(CPUE) = \mu + \alpha_{year} + \beta_{season} + \varphi_{holder} + \gamma_{zone} + \eta_{year \cdot season} + \delta_{year \times zone} + \varepsilon_1$$

where μ is the intercept; *year* is a categorical variable associated with a year (i.e., abundance) effect (1980–1998); *season* is a cat-

TABLE 3.

FIAS-based abundance estimates expressed as the mean number (and associated standard error) of abalone per 60 m^2, calculated by averaging over the number of 30 m × 2 m transects indicated.

	Stations 1–6 = poached area Stations 7–20 = nonpoached area				
	Year				
Zone C All (n = 20)	1995	1996	1997	1998	1999
Mean no. per 30 m transect	18.50	16.40	13.70	10.30	7.80
Standard error	4.00	4.20	6.40	3.40	2.20
Poached area (n = 6)					
Mean no. per 30 m transect	22.30	14.00	1.30	1.80	2.00
Standard error	10.00	5.70	0.50	1.20	1.00
Nonpoached area (n = 14)					
Mean no. per 30 m transect	16.80	17.40	19.00	13.90	10.30
Standard error	4.00	5.70	8.90	4.50	2.90

TABLE 4.

Nominal (in kg/min) and GLM-standardized CPUE series from 1980 to present calculated from the "poached" CP and "nonpoached" CNP subareas of Zone C.

	Nominal		Standardized	
Fishing Year	CNP	CP	CNP	CP
1980	1.281	1.326	0.804	0.845
1981	1.409	1.326	0.937	0.900
1982	1.337	1.316	0.918	0.880
1983	1.425	1.358	0.894	0.893
1984	1.444	1.374	0.892	0.892
1985	1.421	1.479	0.875	0.949
1986	1.590	1.688	0.965	1.106
1987	1.727	1.587	1.027	0.979
1988	1.805	1.725	1.010	1.041
1989	1.779	1.711	1.161	1.177
1990	2.091	1.856	1.295	1.219
1991	1.829	1.655	1.084	1.014
1992	1.847	1.888	1.184	1.166
1993	1.645	2.064	1.141	1.405
1994	1.825	2.062	1.087	1.143
1995	1.856	1.765	1.095	1.069
1996	1.430	1.363	0.975	0.961
1997	1.228	1.050	0.770	0.692
1998	1.425	1.176	0.886	0.667

Fishing years are taken to run from October to September. Note that the reason the standard values are rather lower in absolute terms than the nominal values is that the former have been standardised in terms of entitlement holder no. 1, who happens to be one of the less efficient operators.

TABLE 5a.

Catch at age matrix for commercial catches from zone C (poached area) calculated for standardized fishing years indicated.

	Ages						
F/Season	9	10	11	12	13	14	15+
1981	0.293	0.260	0.155	0.100	0.069	0.028	0.095
1982	0.294	0.237	0.139	0.105	0.056	0.019	0.150
1983	0.249	0.253	0.157	0.105	0.062	0.031	0.144
1984	0.198	0.281	0.168	0.113	0.071	0.027	0.141
1985	0.173	0.237	0.158	0.108	0.079	0.026	0.219
1986	0.192	0.316	0.217	0.096	0.071	0.016	0.092
1987	0.141	0.249	0.202	0.133	0.092	0.043	0.141
1988	0.123	0.241	0.189	0.122	0.106	0.040	0.179
1989	0.087	0.228	0.187	0.136	0.104	0.043	0.216
1990	0.140	0.244	0.169	0.139	0.097	0.037	0.175
1991	0.173	0.240	0.200	0.132	0.080	0.026	0.150
1992	0.149	0.240	0.182	0.145	0.092	0.037	0.156
1993	0.176	0.273	0.202	0.122	0.071	0.027	0.130
1994	0.187	0.231	0.213	0.138	0.078	0.027	0.127
1995	0.177	0.162	0.179	0.159	0.088	0.041	0.254
1996	0.040	0.085	0.125	0.121	0.090	0.046	0.492
1997	0.163	0.154	0.134	0.102	0.062	0.027	0.356
1998	0.111	0.139	0.126	0.107	0.069	0.027	0.421

egorical variable associated with the season effect (1 = Jan-Mar; 2 = Apr-Jun; 3 = Jul-Sep; 4 = Oct-Dec); *holder* is a categorical variable associated with the entitlement-holder (1–84); *zone* is a categorical variable associated with the different zones/subareas (A, B, CP, CNP, and D); *year* × *season* is the interaction between years and seasons; *year* × *zone* is the interaction between years and zones/subareas; and ε is the error term, that is assumed to follow a normal distribution. The CPUE values for each zone/subarea were weighted in proportion to the relative area of kelp habitat (data from Tarr 1993).

TABLE 5b.

Catch at age matrix for commercial catches from zone C (nonpoached area) calculated for standardized fishing seasons indicated.

	Ages						
F/Season	9	10	11	12	13	14	15+
1981	0.078	0.188	0.087	0.129	0.101	0.041	0.375
1982	0.051	0.069	0.093	0.109	0.108	0.025	0.545
1983	—	—	—	—	—	—	—
1984	—	—	—	—	—	—	—
1985	0.243	0.286	0.132	0.079	0.073	0.025	0.163
1986	0.137	0.250	0.206	0.140	0.087	0.020	0.195
1987	0.137	0.248	0.226	0.165	0.105	0.046	0.073
1988	0.063	0.248	0.230	0.163	0.122	0.046	0.128
1989	0.075	0.298	0.181	0.144	0.131	0.020	0.150
1990	0.074	0.197	0.150	0.126	0.101	0.034	0.319
1991	0.100	0.185	0.188	0.146	0.113	0.047	0.222
1992	0.045	0.104	0.118	0.134	0.105	0.053	0.441
1993	0.137	0.175	0.161	0.115	0.080	0.031	0.301
1994	0.074	0.137	0.163	0.148	0.094	0.044	0.340
1995	0.089	0.151	0.142	0.127	0.079	0.042	0.370
1996	0.045	0.096	0.110	0.116	0.081	0.042	0.509
1997	0.152	0.192	0.195	0.145	0.069	0.040	0.207
1998	0.149	0.179	0.161	0.137	0.103	0.039	0.233

Proportions in each age class calculated using age-slicing matrix from Moloney (1999). Note: data lacking for years 1983 and 1984.

The Age-Structured Production Model

The age-structured production model (ASPM) applied is detailed in the Appendix. Because different sectors of the fishery exhibit different selectivity patterns with age, the following four sectors are explicitly differentiated in the model: the commercial fishery sector (mostly offshore); the recreational sector (mostly inshore); the poaching/illegal sector (mostly inshore) and the fishery-independent survey (inshore).

The two subareas constituting zone C are assumed to have identical values for resource dynamics parameters, such as the natural mortality rate M, but to have different catch histories. Two separate pre-exploitation spawning biomasses ($B_0^{yr}[CP]$ and $B_0^{yr}[CNP]$) are estimated in the model. Abalone are broadcast spawners (Tarr 1993), and although it is presently not known to what extent larval mixing occurs throughout the main fishing area, larval and postlarval stages are most likely retained in areas close to the parental population with some larval interchange between adjacent areas. McShane (1992) presented evidence for localized dispersal of larval *Haliotis rubra*. In this initial formulation of the model, the recruitment levels for each of the two zone C subareas are calculated separately. This aspect is explored further in future work.

The annual zone C catch by the illegal sector C_y^{poach} is modeled by assuming that C_y^{poach} increases linearly from a value of zero in 1991 to a maximum value of $CP_{\max}$ tons in 1995, whereafter C_y^{poach} stays constant at this value. The assumptions implicit in this representation are based on both admissions by spokespersons for the poaching sector and well as data from police and fisheries enforcement officers on confiscations of abalone (A. Mackenzie pers. comm.). A separate (year-independent) model parameter p_{poach} defines the relative proportions of the poached catch taken from each of the two subareas. The base-case model assumes that a fixed low proportion ($p_{poach} = 0.1$) of the poaching catch originates from the "nonpoached" subarea. The sensitivity of the model to this assumption is tested.

The minimum legal size limit of abalone is a shell width of 114 mm, which corresponds to an age of 8 y (Tarr 1995). Analysis of confiscated abalone samples recovered from the illegal sector (Table 5d) suggests that the minimum age of individuals caught by this sector is approximately 4–5 y. A particular advantage of using an age-structured (or length-structured) model to assess the dynamics of the resource is that proper account is taken of the fact that the illegal catch component includes animals of sublegal size, which can have important consequences for resource trends. The base-case selectivity functions applied in the model, and the procedure applied to estimate the values of their parameters, are described in the Appendix.

RESULTS

GLM Model Implementation

Commercial data obtained from divers between the years 1980 and 1998 were used to perform the GLM analysis. Following the removal of outliers (1% of total) based upon observations with large residuals in an initial GLM fit, a total of 34–189 data points remained for the analysis. The residuals for the subsequent GLM

TABLE 5c.

Catch at age matrix from FIAS data for the "poached" and "nonpoached" areas of zone C for each of the standardized fishing seasons indicated.

Poached Area—Mudge Point	Proportion in Age Class							
	5	6	7	8	9	10	11	12+
1995	0.049	0.260	0.242	0.154	0.107	0.065	0.048	0.076
1996	0.063	0.268	0.294	0.148	0.066	0.048	0.049	0.066
1997	0.048	0.508	0.111	0.000	0.000	0.000	0.111	0.222
1998	0.000	0.182	0.182	0.000	0.182	0.050	0.132	0.273
Nonpoached area—Hermanus								
1995	0.112	0.200	0.199	0.158	0.140	0.046	0.029	0.116
1996	0.097	0.246	0.235	0.148	0.139	0.048	0.007	0.080
1997	0.117	0.313	0.219	0.216	0.058	0.022	0.035	0.019
1998	0.077	0.193	0.250	0.203	0.158	0.044	0.021	0.054
1999	0.081	0.262	.250	0.137	0.130	0.048	0.036	0.056

Proportions in each age class calculated using age-slicing matrix from Moloney 1999. Note there are currently no data for the poached area in 1999. The proportions in age classes 12 to 15+ have been lumped together to reduce the number of categories containing a proportional abundance less than 2% in any one year.

TABLE 5d.

Catch at age matrix for the poached data from zone C, modified from Moloney (1999).

Year	Ages							
	5	6	7	8	9	10	11	12+
1995	0.007	0.045	0.121	0.172	0.217	0.167	0.095	0.175
1996	0.075	0.228	0.291	0.186	0.106	0.053	0.014	0.046
1997	0.095	0.115	0.135	0.119	0.106	0.089	0.054	0.287
1998	0.080	0.139	0.215	0.186	0.158	0.085	0.049	0.089

Note "year" is calendar year not fishing year, but as explained in the text these are assumed equivalent for present purposes. The proportions in age classes 12 to 15+ have been lumped together to reduce the number of categories containing a proportional abundance less than 2% in any one year.

were further examined to check, in particular, for evidence of heteroscedasticity. Figure 3 shows evidence for larger residuals associated with lower effort. To account for this heteroscedasticity, the iterative procedure used by Glazer (1999) was used to develop a weighted GLM, where the weights are the inverse of the variance, $1/\sigma_i^2$, where the assumption is made that $\sigma_i^2 = a + b/E_i$, with E_i the effort (dive time) for observation i. The resultant maximum likelihood estimates for a and b are 0.8 and 20.5 min, respectively. Full results of the GLM analysis are presented in Plagányi and Butterworth (2000). A summary of the increases in r^2 achieved as the explanatory variables were added to and removed from models in turn is presented in Table 6. Approximately 43% of the total variation is explained by the model. The nominal and standardized CPUE indices are shown in Table 4, and Figure 4 shows a comparison between the standardized CPUE trends computed for the two subareas CP and CNP.

The ASPM

Model parameter estimates as well as log-likelihood contributions are summarized in Table 7, for the base-case scenario and for each of four preliminary sensitivity tests. Sensitivity scenario I tests the effect of reducing the value of the parameter h, which determines the "steepness" of the stock–recruit relationship, from 0.7 to 0.6. This direction of change was specifically chosen, because it effectively reduces the fraction of pristine recruitment that results when spawning biomass drops; i.e., increases the possibility of recruitment overfishing. Sensitivity scenario II is a single test of changing the assumption that only a small proportion ($p_{poach} = 0.1$) of the total zone C poaching takes place in subarea CNP. Sensitivity scenario III provides an example of model results under the assumption that natural mortality M is age dependent (versus age independent, as in the base-case). The age-specific natural mortality M_a in this scenario was estimated using the following simplified functional form:

$$M_a = c_1 + \frac{c_2}{a+1} \qquad (1)$$

where constant c_1 is estimated in the model-fitting process, and constant c_2 set equal to 0.3 for present purposes. Finally, sensitivity scenario IV shows the base-case results obtained before down-weighting the catch-at-age $-\ln L$ contributions as detailed in the Appendix.

The base-case scenario presented in this paper is intended purely for illustrative purposes, and the fits obtained for each subarea to both the CPUE data and the FIAS data are shown in Figures 5a and b. Model results suggest a pristine spawning biomass, B_0^y, of 1,580 and 3,360 tons, respectively, for subareas CNP and CP. The base-case estimate of M is 0.15 yr^{-1}.

The base-case selectivity estimates are illustrated in Figure 6. The estimated commercial and recreational selectivity functions reflect the fact that the minimum legal size corresponds to an age of approximately 8 y; whereas, the estimated poaching selectivity function reflects the fact that sublegal animals are caught. Because the FIAS transects are situated inshore, the estimated FIAS selectivity function (Fig. 6) concurs with the observation of Tarr (1993) that the mean size of *H. midae* increases with depth.

The model estimated an initial steep decline in the spawning biomass of abalone, particularly in subarea CP (Fig. 7), as a result

TABLE 5e.

Catch at age matrix for the Recreational data from Zone C, modified from Moloney (1999).

		Ages							
		8	9	10	11	12	13	14	15+
Zone C	1992	0.013	0.208	0.217	0.164	0.115	0.088	0.031	0.164
	1993	0.011	0.210	0.254	0.174	0.112	0.080	0.030	0.130
	1994	0.005	0.128	0.250	0.209	0.163	0.089	0.027	0.130
	1995	0.001	0.168	0.269	0.206	0.161	0.076	0.026	0.093
	1996	0.005	0.184	0.250	0.176	0.130	0.070	0.027	0.159
	1997	0.014	0.270	0.280	0.161	0.094	0.056	0.021	0.102
	1998	0.000	0.136	0.249	0.194	0.131	0.101	0.131	0.158
	1999	0.016	0.212	0.243	0.167	0.135	0.092	0.023	0.112
Mudge Point (subarea CP)									
	1997	0.067	0.145	0.126	0.103	0.100	0.066	0.036	0.357

Note that the "year" indicated refers to a fishing year as defined in the model.

of the high historic exploitation levels in the 1960s (Fig. 8). A slight recovery of the stock level is estimated to have occurred during the 1980s, followed by a relatively stable trend in subarea CNP and a marked downward trend in subarea CP in recent years. The estimate of $CP_{\max}$ furnished by the base-case model was 240 tons, which is greater than the commercial take during the 1990s. Based on the results of the base-case model scenario, the current spawning biomasses of abalone in the "nonpoached" and "poached" areas of zone C are estimated at *ca.* 85% and 17%, respectively, of their pre-exploitation levels, expressed in terms of the spawning biomass B_0^y.

Assuming commercial selectivity, the estimated maximum sustainable yields MSY for abalone are 136 and 289 tons per annum, respectively, for subareas CNP and CP (Table 7; Fig. 8), with a combined zone C estimate of 425 tons per annum and $F_{MSY} = 0.28$. However, if sublegal animals are caught, sustainable yields will be less than this. The estimated MSY assuming poaching selectivity for abalone in zone C is 272 tons per annum (Fig. 8). Assuming poaching selectivity, comparison of the best estimate of MSY with total catch estimates (Fig. 8), suggests that catches probably exceeded replacement yields from about 1993, resulting in a decrease in stock biomass.

Comparisons between observed and model-predicted catch-at-age estimates are illustrated graphically in Figure 9. For ease of viewing, the observations and estimates have been averaged over all years for which data exist.

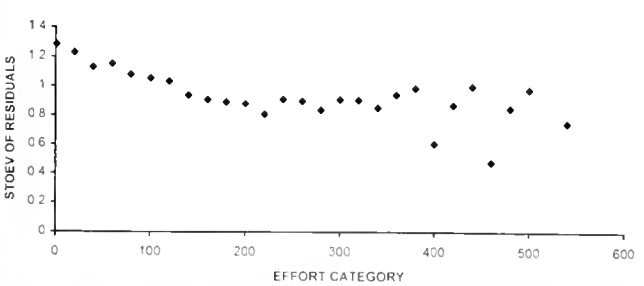

Figure 3. Plot of the standard deviation of the residuals (corresponding to an initial GLM fit of the data) versus effort (min). To take account of this heteroscedasticity, a weighted GLM was developed as described in the text.

DISCUSSION

The same general pattern of an increase in standardized catch rates during the 1980s, followed by a decrease during the 1990s is evident in both subareas (Fig. 4). The observed decline in both catch rates and the fishery independent abundance index (Fig. 2) in subarea CP is particularly steep, and it has been recognized for some time that continued depletion of the resource in zone C is inevitable, unless catches by all sectors continue to be drastically reduced (e.g., Plagányi & Butterworth 1999). Considering that total catches in zone C in the 1960s were substantially greater than those taken in the 1970s and 1980s (Fig. 8) (this hardly seems likely not to have been the case, although the details of the zonal-partitioning of catches assumed above could be in error to some extent), it is not too surprising that the CPUE trend shows an increase toward the end of the 1980s (Fig. 4). This is the obvious explanation for the observed increase in CPUE estimates during the 1980s, despite perceived increases in over-all catch levels over

TABLE 6.

Summary of the results of the GLM analysis showing r^2 as a function of sequentially including the variables shown below in the model.

Model variables	r^2
s	0.075
z	0.102
h	0.162
y	0.144
y, z	0.225
y, s	0.168
y, h	0.286
y, h, s	0.307
y, h, z	0.352
y, h, z, s	0.369
y, h, z, s, $y^* z$	0.411
y, h, z, s, $y^* s$	0.398
y, h, z, s, $y^* z$, $y^* s$	0.426

The variables are abbreviated as follows: y = year (1980–1998), s = season (1–4), z = zone/subarea (A, B, CNP, CP, D), h = entitlement holder (1–84).

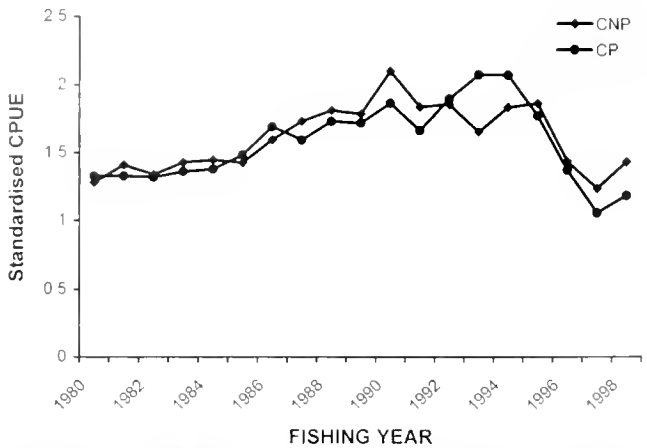

Figure 4. Comparison between the standardized catch-per-unit-effort (CPUE) trends for the "poached" (CP) and "nonpoached" (CNP) subareas of zone C. Values are derived from an iterative effort weighted GLM (see text).

this period (Tarr 1993). It is to be expected from a relatively slow-growing long-lived resource afforded a respite as high and unsustainable historic catch levels are reduced substantially to below the then current sustainable yields. If we assume that the CPUE trend in zone C is a reasonable index of stock abundance, this suggests that; whereas, the zone C commercial take during at least some of the 1970s and 1980s was below sustainable yield (SY) levels, total catches during the 1990s have again exceeded such yields, resulting in a concomitant decline in CPUE values over this most recent period. This recent decline in CPUE is fully consistent with catch data only if the latter are considered to include the considerable poaching component of the over-all catches over recent years as estimated by the model.

The similarities in the catchability coefficient estimates $\hat{q}^{CPUE}[CP]$ and $\hat{q}^{CPUE}[CNP]$ (Table 7) for the two subareas are as expected, given the approximately equal habitat areas and the similarities in the standardized catch rates over much of the 1980s (Fig. 4). The difference in the pristine spawning biomass estimates $B_0^{yp}[CNP]$ and $B_0^{yp}[CP]$ (Table 7) are, in the main, attributable to the partitioning of the historic zone C catch data between the two subareas.

The estimated poaching catches are substantial in all of the scenarios investigated in this analysis (Table 7), despite the fact that the recent steep decreases in the FIAS data are not adequately reproduced by any of the model scenarios. It is important to note that the inability of the model to simulate the trends in the FIAS data successfully, particularly the steep decline in subarea CP, suggests that pending further analyses, model estimates are likely to be positively biased.

The inadequate fit of the model to the FIAS data (Fig. 5) may, in part, be explained by the fact that since 1994, there has been a dramatic decrease in young abalone (5–40 mm) found during diving surveys in zones C and D (Tarr et al. 1996). This has been ascribed to a decrease in the abundance of sea urchins (in turn caused by an increase in the abundance of the rock lobster—an important predator on urchins). Young abalone shelter and feed under sea urchins; hence, it has been proposed that these multispecies interactions are resulting in substantially reduced abalone recruitment (Tarr et al. 1996). This factor is ignored in the current model, but needs to be investigated further (see Future Work). It should be noted, however, that only animals older than approximately 5 y are sampled on the FIAS transects; thus, there is at least a 5-y time lag before the effects of a recruitment failure are manifest in the data.

Comparisons of the log-likelihood contributions for the base-case and scenarios I–IV (Table 7) suggest that the main conflict in the model fits is between the fit to the CPUE data for the poached subarea and the catch-at-age data for the recreational sector. FIAS (poached subarea in particular, see, e.g., Fig. 9) and poaching sector to a lesser extent. In these instances, an improved fit to the CPUE data (i.e., a decrease in the log-likelihood and $\hat{\sigma}$ values) results in a sacrifice to the fit to the catch-at-age data. The fact that the CPUE (CP) and age data are pushing in different directions requires further consideration.

The base-case estimates of the ratios $B_0^{yp}(98)/B_0^{yp}(53)$ are 0.85 and 0.17 for subareas CP and CNP, respectively, but these estimates varied substantially across the various model scenarios. This stresses the importance of refining the estimate of h, for example, as well as investigating further the model's sensitivity to the assumption that natural mortality is age independent. The use of an age-dependent mortality function versus an age-independent mortality value is particularly important because of, among other things, the sensitivity of the estimated MSY values to this assumption (Table 7).

The results discussed above follow only given the CPUE data, but the appropriateness or otherwise of using CPUE data as an index of abalone stock abundance has in the past been questioned both in the South African context and elsewhere in the world. There are a number of reasons why CPUE may be a reasonable index of abalone stock abundance in the South African context but not necessarily elsewhere in the world. The major South African abalone fishery is located in shallow kelp bed areas, relatively close inshore, along a stretch of coastline measuring less than a hundred kilometers and with relatively easy access to most of the areas (R. Tarr pers. comm.). Furthermore, the 10 or so launching sites currently in use along this coastline are exactly the same sites that were in use during the 1950s when the fishery began; hence, fishers have fished fairly evenly throughout the area. Even if very large aggregations existed in the past, the resource was so heavily fished during the 1960s that these would since have been reduced substantially; hence, the effect of spatial patchiness dampened to a large extent. Moreover, fishing effort is spread over most of the major fishing zones so that the general pattern of diver behavior is not one in which new aggregations are sought out and then sequentially depleted so that catch rates remain fairly stable, despite the resource abundance declining, as has been demonstrated for fisheries off Australia (Shepherd & Baker 1998), California (Tegner et al. 1989), and Mexico (Prince & Guamán del Próo 1993). It is now well established that in such areas as South Australia and Tasmania, the spatial variation in density is such that, coupled with the added complexities of diver behavior, CPUE cannot be considered a reliable indicator of stock abundance (Breen 1986, Prince 1992, Shepherd et al. 1992, Keesing & Baker 1998, Worthington & Andrew 1998). As proposed by Dichmont et al. (2000) one option available to resource managers with some confidence in CPUE as an index of abundance for their fishery, is to use a dynamic model tuned to both CPUE data and also a survey index.

The FIAS data series not only provide a valuable independent index of abundance but are also useful, because they include information on abalone from age 5 upward compared with the CPUE data, which captures information on animals aged approximately 8 y or more. This means that the FIAS abundance index is

TABLE 7.

Best fit estimates of the pre-exploitation spawning biomass B_0^{sp} for the "poached" CP and "nonpoached" CNP areas of zone C, the estimated natural mortality estimate M and the poaching maximum CP_{max}, where the proportion of CP_{max} from area CNP is fixed at 0.1 in the base-case.

	Base-Case	Sens. 1 h	II p_{poach}	III Age-Dependence of M	IV (No Downweighting)
h	0.7	0.6	0.7	0.7	0.7
p_{poach}	0.1	0.1	0.3	0.1	0.1
Wt Age	0.1	0.1	0.1	0.1	1
B_0^{sp} [CNP]	1584	1745	2689	6123	1601
B_0^{sp} [CP]	3358	3457	3422	6303	3404
q^{CPUE} [CNP]	2.21×10^{-6}	1.85×10^{-6}	0.98×10^{-6}	1.04×10^{-6}	$2.03 + 10^{-6}$
q^{CPUE} [CP]	1.28×10^{-6}	1.55×10^{-6}	1.34×10^{-6}	1.72×10^{-6}	1.10×10^{-6}
M	0.154	0.174	0.17	M(a) = 0.23 + 0.3/a + 1	0.24
CP_{max}	242	250	359	242	203
$\hat{a}$ (CS)	8.983	9.047	8.857	9.654	9.404
$\hat{a}$ (RS)	9.376	8.796	8.997	9.195	9.020
$\hat{a}$ (PS)	6.588	6.622	6.386	5.533	6.750
$\hat{a}$ (FS)	5.549	5.489	5.466	5.617	5.513
μ (CS)	0.007	0.006	0.006	0.005	0.009
μ (RS)	0.005	0.006	0.005	0.004	0.006
μ (PS)	0.141	0.148	0.120	0.127	0.136
μ (FS)	0.059	0.048	0.052	0.063	0.073
δ (CS)	2.189	1.435	17.41	1.391	1.265
δ (RS)	1.097	2.510	1.143	2.053	2.433
δ (PS)	1.212	1.145	1.228	1.149	1.477
δ (FS)	1.032	1.110	1.516	1.758	1.727
$-\ln L$ CPUE (CNP) [$\hat{\sigma}^{CS}$]	−27.863 [0.14]	−27.958 [0.14]	−27.437 [0.14]	−25.996 [0.15]	−27.413 [0.14]
$-\ln L$ CPUE (CP) [$\hat{\sigma}^{CS}$]	−38.477 [0.08]	−37.490 [0.08]	−37.791 [0.08]	−30.408 [0.12]	−30.992 [0.12]
$-\ln L$ FIAS (CNP)	−4.729	−4.686	−4.873	−4.575	4.585
$-\ln L$ FIAS (CP)	13.661	13.830	13.748	14.186	14.896
$-\ln L$ age CS (CNP) [$\hat{\sigma}_c^{CS}$]	−6.671 [0.12]	−6.691 [0.12]	−5.793 [0.13]	−7.127 [0.12]	−63.759 [0.13]
$-\ln L$ age CS (CP) [$\hat{\sigma}_c^{CS}$]	−11.898 [0.09]	−11.553 [0.09]	−11.677 [0.09]	−9.280 [0.11]	−105.598 [0.10]
$-\ln L$ age RS [$\hat{\sigma}$] (combined)	−1.495 [0.14]	−1.481 [0.14]	−0.899 [0.16]	−3.111 [0.09]	−38.441 [0.08]
$-\ln L$ age RS (CP) [$\hat{\sigma}$]	0.246 [0.22]	0.239 [0.22]	0.099 [0.21]	0.186 [0.21]	1.413 [0.21]
$-\ln L$ age FIAS (CNP) [$\hat{\sigma}$]	−1.941 [0.11]	−1.781 [0.12]	−1.816 [0.12]	0.299 [0.21]	−19.152 [0.11]
$-\ln L$ age FIAS (CP) [$\hat{\sigma}$]	−2.461 [0.14]	−5.023 [0.09]	−1.600 [0.16]	−6.409 [0.07]	−73.650 [0.06]
$-\ln L$ age PS [$\hat{\sigma}$]	−0.792 [0.08]	−0.600 [0.09]	−1.117 [0.05]	−0.411 [0.12]	−2.602 [0.14]
$-\ln L$ (Total)	−82.421	−83.195	−79.156	−72.645	−349.815
B_0^{sp} (98)/K [CNP]	0.85	0.58	0.64	0.39	0.69
B_0^{sp} (98)/K [CP]	0.17	0.35	0.39	0.23	0.62
MSY [CNP]	136	153	231	376	193
MSY [CP]	289	304	295	387	410

Minimum values of the negative of the log-likelihood function and estimated current depletion are also shown. The estimated selectivity parameters are shown for the commercial sector (CS), recreational sector (RS), poaching sector (PS) and FIAS (F). Values of σ are given in square brackets alongside the corresponding log-likelihood values.

more sensitive than the CPUE index (cf. Figs. 2 and 4) to changes in the younger age classes. This is particularly important in zone C, where extensive poaching of sublegal abalone individuals as well as a hypothesized multispecies effect (Tarr et al. 1996) have caused a rapid decline in the numbers of small individuals. Consequently, the FIAS data have indicated a more negative scenario than have the CPUE data and are, thereby, providing valuable clues as to the factors to consider in modeling the resource.

Finally, we reiterate that the inferences above are based on preliminary model results only. Coefficients of variation and confidence intervals remain to be calculated for the model estimates, and the sensitivity of the model to its assumptions and parameters must be examined. This paper is not, and does not pretend to be a thorough and complete analysis. Rather it is intended as a description of the stock assessment approach being applied in South Africa, motivated by the fact that it currently differs from approaches in practice elsewhere in the world for abalone resources.

Planned Future Work

Some of the issues to be addressed in future work are outlined below.

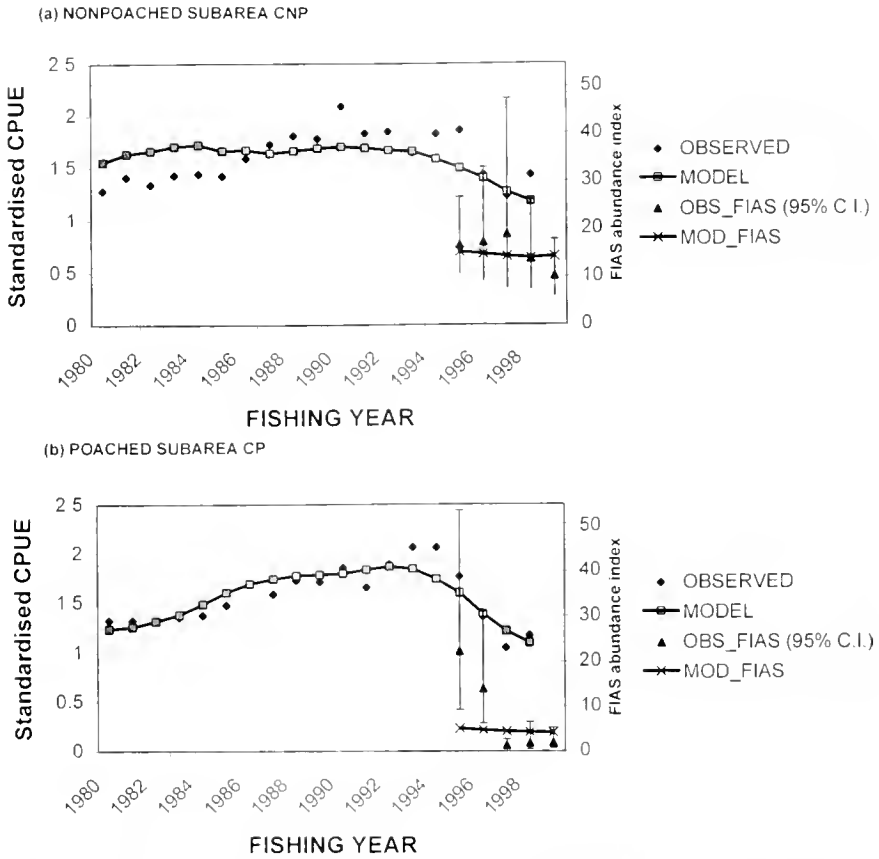

Figure 5. Comparisons between the standardized CPUE and model-predicted CPUE values (from the base-case scenario) for each of subareas CNP (a) and CP (b). The base-case model fit to the FIAS data is also shown. The 95% confidence intervals shown for the observed FIAS data were calculated as $\bar{x} * e^{\pm 1.96 \cdot CV}$.

Sensitivity to variability/changes in the following assumptions must be investigated:

1. the proportions of the all-zone annual commercial catches before 1977 allocated to zone C (currently a fixed proportion of 27% for all such years) as well as the subsequent reallocation of these catches between subareas CNP and CP (24%: 76%, respectively, in the base-case model);
2. the years in which substantial poaching catches commenced (currently 1991), and reached their maximum (currently 1995);
3. the age at 100% sexual maturity (currently $a_m = 7$ yrs);
4. stock-recruitment function parameters (currently $h = 0.7$);
5. alternative choices of growth parameters (current values given in the Appendix);
6. trends in catchability (q) over time that could have resulted from improved efficiency; this could be effected by adjusting the input CPUE values by a multiplicative factor $e^{-\lambda y}$, where y is the year, and λ is a constant, with results being contrasted for different choices of λ; and
7. the assumed linear relationship between CPUE and biomass;

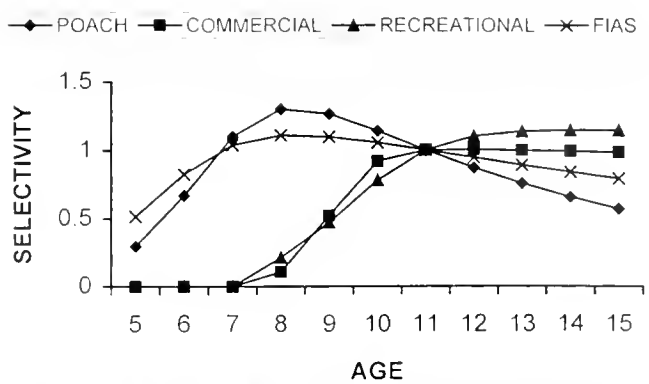

Figure 6. Plots of the base-case selectivity functions estimated for the commercial, recreational, and poaching fishery sectors, and for FIAS. A description of the general functional form used is given in the Appendix and the fitted parameter values listed in Table 7.

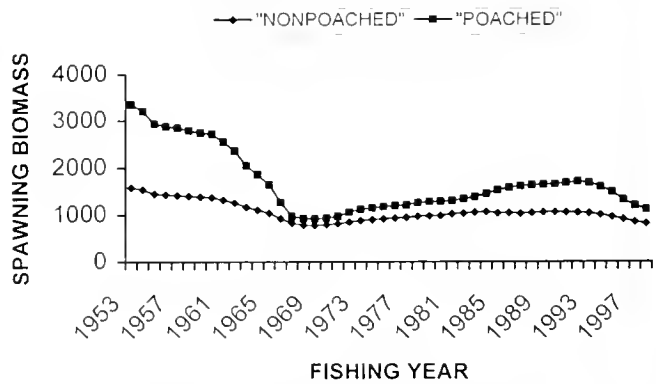

Figure 7. The base-case trend in the spawning biomass (tons) of abalone in the "nonpoached" (CNP) and "poached" (CP) subareas of zone C. The spawning biomass includes all animals older than a_m, the age corresponding to 100% sexual maturity (see Appendix).

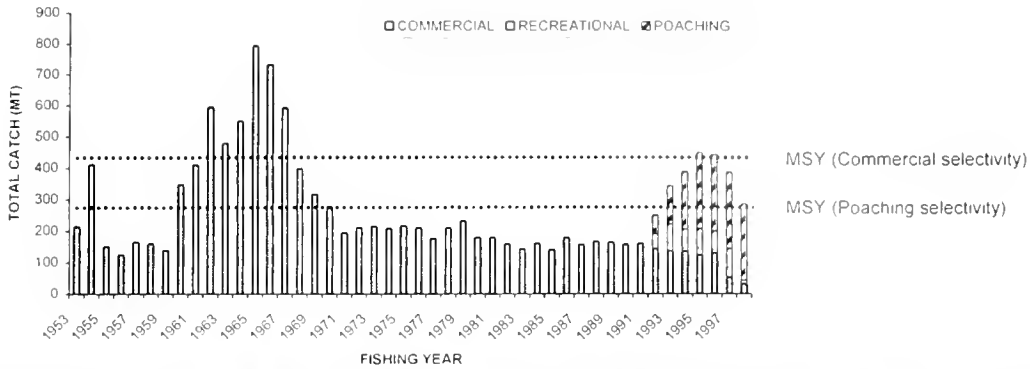

Figure 8. Comparison between the base-case zone C maximum sustainable yield (MSY) (tons) estimates [assuming either a commercial or a poaching selectivity function (see Fig. 6)] and total annual abalone catches (tons) over the period 1953 to 1998. The total catches shown include both the commercial and recreational catches from subareas CP and CNP combined, as well as the total poaching catch estimated in the base-case model.

this could be examined by, for example, exploring the consequences of an assumption such as CPUE proportional to square root (biomass), which implicitly incorporates the saturation effect possibilities raised by several authors.

In addition, likelihood-profile-based estimates of confidence intervals for model-estimated parameters must be calculated.

The model has been substantially revised from that applied in an earlier assessment to the abalone resource in the other main fishing zones (e.g., Plagányi & Butterworth 1999) and the revised version, therefore, must be applied to the other zones. This is not necessarily straightforward, because previous experience has shown that the data concerned do not necessarily support the es-

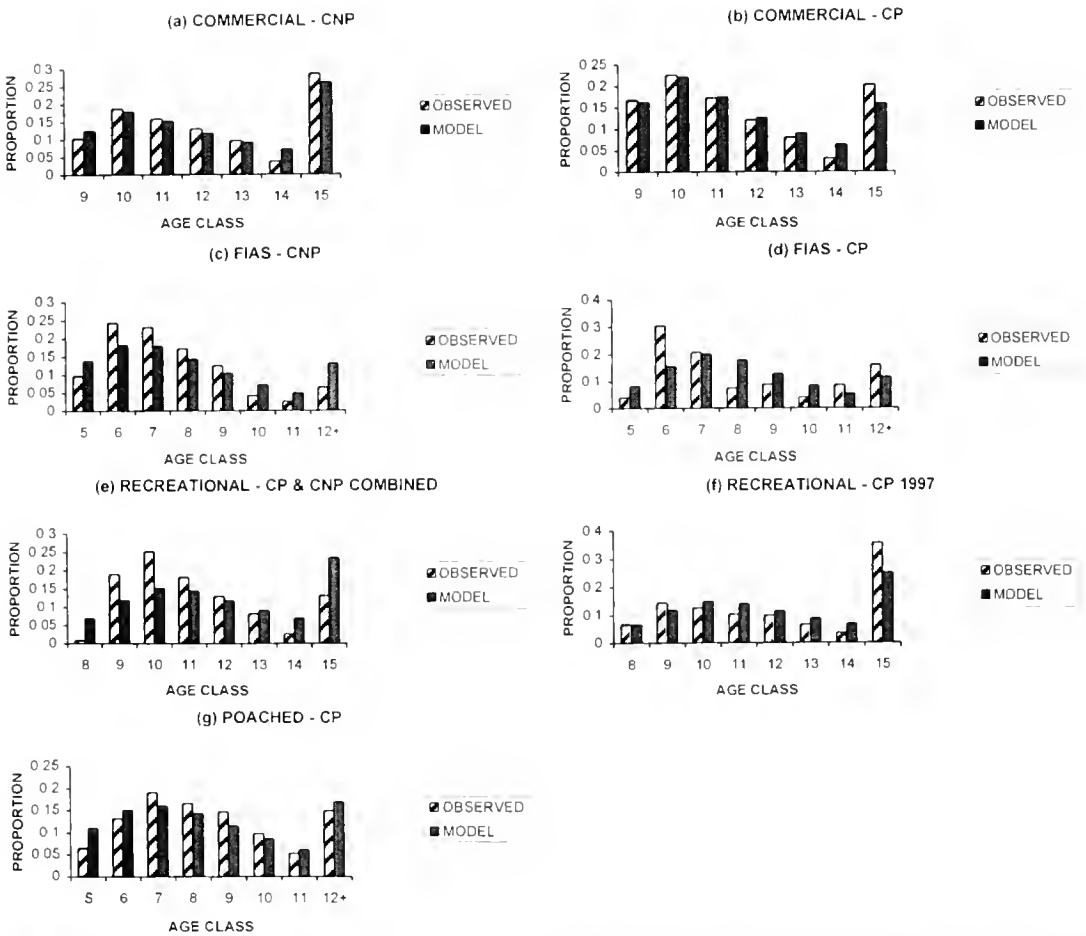

Figure 9. Plots showing the observed versus model-predicted catch-at-age estimates from FIAS and from the commercial, recreational, and poaching sectors. In all instances (except the 1997 recreational data, which is a single year's worth of data), the observations and estimates have been averaged over all years for which data exist (see Tables 5 a–e). The observed catch-at-age proportions were derived from length distribution data that was cohort-sliced using a von Bertalanffy growth curve (Tarr 1995).

timation of as many parameters, so that M (for example) might have to be fixed externally by using the estimate for zone C.

Matters to be pursued in the longer term include the following:

1. computing probability distributions for biomass projections under different future catch scenarios, taking uncertainty in the estimates of the model parameters B_0^{yr}, CP_{max}, and M into account;
2. investigating alternative functional forms for the stock recruitment relationship assumed as well as testing sensitivity to possible depensation effects at low biomass levels;
3. investigating various hypotheses as to the extent of mixing of the larval products throughout the commercial fishery zones;
4. consideration of the possibility (indeed, probability) that natural mortality M is not independent of age—the data are unlikely to support direct estimation of this age dependence, so this investigation will likely need to consider different external specifications; and
5. allowance for multispecies effects, such as a postulated recruitment decrease as a consequence of urchin reductions (Tarr et al. 1996), might be investigated. However, thorough prior discussion is first needed about whether there is a sound basis in the available data to distinguish the alternative hypotheses of a systematic trend in urchin numbers over the near 50-year history of the fishery, and of serially correlated fluctuations of an over-all stationary (i.e., trendless) process. If the latter is the case, the existing model may already make adequate implicit allowance for this, because the effects of such recruitment fluctuations could be integrated out given the large number of year classes in the population, although this should be checked by further quantitative evaluations.

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APPENDIX

THE POPULATION MODEL USED FOR ESTIMATING RESOURCE DYNAMICS PARAMETERS AND PROJECTING BIOMASS TRENDS

The model applied is a deterministic age-structured production model.

Dynamics

$$N_{y+1,0} = R(B^{sp}_{y+1}) \tag{A1}$$

$$N_{y+1,a+1} = (N_{y,a}e^{-\frac{M}{4}} - C_{y,a})e^{-\frac{3M}{4}} \qquad 0 \le a \le z-2 \tag{A2}$$

$$N_{y+1,z} = (N_{y,z}e^{-\frac{M}{4}} - C_{y,z})e^{-\frac{3M}{4}} + (N_{y,z-1}e^{-\frac{M}{4}} - C_{y,z-1})e^{-\frac{3M}{4}} \tag{A3}$$

where

- $N_{y,a}$ is the number of abalone of age a at the start of fishing year y,
- $C_{y,a}$ is the total number of abalone of age a taken by the fishery, by recreationals and by poachers in fishing year y,
- $R(B^{sp})$ is the recruitment vs spawner biomass relationship assumed (see below),
- M is the natural mortality (assumed independent of age), and
- z is the largest age considered (i.e. corresponding to a "plus group").

The commercial abalone fishery season currently extends from October to June and a fishing year in the model is assumed to run from October to September. The approximation of the fishery as a pulse catch at the start of each calendar year is here considered of sufficient accuracy given that most of the catch is made over the October–March period (see Table 2), and because the annual catches from this long lived resource are not that large a fraction of the overall biomass. Equations (2) and (3) above have accordingly been modified to reflect the fact that catches are subtracted at the end of the first quarter of the fishing year. As the fisher-independent surveys are only conducted towards the end of the second quarter of the fishing year, comparisons with the observed FIAS data are made at time $y + \frac{1}{2}$ in terms of the model whereas comparisons with the remaining data are made at time $y + \frac{1}{4}$ in the model.

The subsistence sector of the fishery is ignored in the current analysis because it is a very recent participant and although no data on catches currently exist, the impact of this sector is presumed to be small. The subsistence sector could most easily be considered a subcomponent of the recreational sector, as although the subsistence sector is now permitted to sell its catch, they are essentially recreational fishers operating in shallow waters and with a fixed bag limit.

The total number of abalone of age a caught each year ($C_{y,a}$) is given by:

$$C_{y,a} = \sum_s C^s_{y,a} \tag{A4}$$

where s indicates the sector of the fishery (e.g. commercial, recreational, poaching).

The annual catch by mass (C^s_y) for sector s is given by:

$$C^s_y = \sum_{a=5}^{z} w_a C^s_{y,a} \tag{A5}$$

where w_a is the mass of an abalone of age a (to ignore clutter of subscripts, we ignore here that for the plus group mass $w_{y,z}$ is year dependent). The summation is taken from age $a = 5$ as no abalone of a size corresponding to ages below 5 are taken.

The mass-at-age is given by the combination of a von Bertalanffy growth equation and a mass-length relationship (where l refers to shell length in mm) (from Tarr 1995):

$$l(t) = l_\infty[1 - e^{-K(t-t_0)}] \tag{A6}$$

$$w_{y,a} = w(y, t = a) = c[0.913^{3\times}\, l(t) - 11.59]^d \tag{A7}$$

Note that mass-at-age is year-independent for abalone of age $a < z$ and that $w_{y,a+1/4} = w(y, t = a + 1/4)$ is computed for use in calculating the sector-specific exploitable biomasses after the first quarter (see below). However, the mass-at-age for the plus group varies over time, depending on the average age of the plus group in year y, $\bar{z}_y$, which is calculated as described below:

$$\bar{z}_y = \frac{(\bar{z}_{y-1} + 1)(N_{y,z} - C_{y,z})e^{-M} + z \cdot (N_{y,z-1} - C_{y,z-1})e^{-M}}{N_{y+1,z}} \tag{A8}$$

The above is an approximation only (as it ignores, e.g., the fact that catches are subtracted not at the start of the year but at the end of the first quarter of each year) but is considered sufficiently accurate for present purposes.

The sector-specific catch by mass in year y is given by:

$$C^s_y = \sum_{a=5}^{z} w_a N_{y,a} e^{-M \cdot \frac{1}{4}} S^s_a F^s_y \tag{A9}$$

where S^s_a is the fishing selectivity-at-age for sector s (this pattern is assumed not to change over time), and F^s_y is the fishing "mortality" (strictly here that proportion of the numbers present at the start of the calendar year which are caught) at a reference age, assumed for these computations to be $a = 11$ for all sectors. Based on data from A. Mackenzie (Marine & Coastal Management, pers comm), the minimum age of animals assumed caught by the poaching sector is 5 years, so that for this sector $S^s_a = 0$ for $a < 5$. The commercials and recreationals are both assumed not to catch animals below the legal size limit, so that for these sectors $S^s_a = 0$ for $a < 8$.

The sector-specific exploitable ("available") component of abundance is either in terms of exploitable biomass:

$$B^{exp,s}_y = \sum_{a=5}^{z} w_{y+1/4,a} S^s_a N_{y,a} e^{-M \cdot \frac{1}{4}} \tag{A10}$$

or "available" population numbers (in the case of FIAS, which for these purposes can be considered as another fishery sector s):

$$N^{exp,s}_y = \sum_{a=5}^{z} S^s_a N_{y,a} e^{-M \cdot \frac{1}{2}} \tag{A11}$$

The summation is from age $a = 5$ as only animals larger than 100 mm shell length are recorded so as to reduce uncertainty in the estimates due to the non-emergent/cryptic behaviour of juveniles. This corresponds to a minimum sampling age of approximately 5 years, so that for this sector $S^s_a = 0$ for $a < 5$.

The proportion of the resource harvested each year (F_y^s) by sector s is therefore given by:

$$F_y^s = C_y^s / B_y^{exp,s} \tag{A12}$$

where

$$C_{y,a}^s = S_a^s F_y^s N_{y,a} e^{-M \cdot 4} \tag{A13}$$

The annual zone C catch by the illegal sector C_y^{poach} is modelled by assuming that C_y^{poach} increases linearly from a value of zero in 1991 to a maximum value of $CP_{\max}$ tonnes in 1995, whereafter C_y^{poach} stays constant at this value. A separate (year-independent) model parameter p_{poach} defines the relative proportion of the poached catch that originates from the "nonpoached" subarea.

Spawning biomass—recruitment relationship

The spawning biomass of each subarea in year y is given by:

$$B_y^{sp} = \sum_{a=a_m}^{z} w_a N_{y,a} \tag{A14}$$

where a_m is the age corresponding to 100% sexual maturity, which is assumed here to be described by a knife-edge function with age.

The number of recruits in each of the two subareas at the start of fishing year y is related to the spawner stock size by a stock-recruitment relationship. A Beverton–Holt form is assumed, i.e.:

$$R(B_y^{sp}) = \frac{\alpha B_y^{sp}}{\beta + B_y^{sp}} \tag{A15}$$

In order to work with estimable parameters that are more meaningful biologically, the stock-recruit relationship is re-parameterised in terms of the pre-exploitation equilibrium spawning biomass, B_0^{sp}, and the "steepness" of the stock-recruit relationship, where "steepness" is the fraction of pristine recruitment that results when spawning biomass drops to 20% of its pristine level, ie.

$$hR_0 = R(0.2\ B_0^{sp}) \tag{A16}$$

from which it follows that:

$$h = 0.2[\beta + B_0^{sp}]/[\beta + 0.2\ B_0^{sp}] \tag{A17}$$

and hence:

$$\alpha = \frac{4hR_0}{5h - 1} \tag{A18}$$

and:

$$\beta = \frac{B_0^{sp}(1 - h)}{5h - 1} \tag{A19}$$

Biomass trajectories

Given a value for the pre-exploitation spawning biomass B_0^{sp} of abalone, together with the assumption of an initial equilibrium age structure, we have:

$$B_0^{sp} = R_0 e^{-Ma_m}\left[\sum_{a=a_m}^{z-1} w_a e^{-M(a-a_m)} + w_z e^{-M(z-a_m)}/(1 - e^{-M})\right] \tag{A20}$$

which can be solved for R_0. The initial numbers at age for the projections, corresponding to the deterministic equilibrium, are:

$$N_{0,a} = \begin{cases} R_0 e^{-Ma} & 0 \le a \le z-1 \\ R_0 e^{-Mz}/(1 - e^{-M}) & a = z \end{cases} \tag{A21}$$

Numbers-at-age for subsequent years are then computed by means of equations (1)–(20).

PARAMETER VALUES

Input parameters:

The following fixed parameter values used in the model are in the main based on estimates presented by Tarr (1993) and Tarr (1995):

$$\begin{aligned} l_\infty &= 173 \text{ mm} \\ \kappa &= 0.186 \text{ yr}^{-1} \\ t_0 &= 0 \text{ yr} \\ c &= 0.002 \text{ gm/cm}^{2.61} \\ d &= 2.61 \\ a_m &= 7 \end{aligned}$$

with the computations assuming a plus group at age $z = 15$ yrs.

Moreover, the base-case assumes that $p_{poach} = 0.1$ and that $h = 0.7$. The base-case value of the steepness parameter h corresponds roughly to the median ($h = 0.74$) of a distribution of h values for stock-recruit functions fitted to the fisheries stock recruitment database developed by R. A. Myers and colleagues (Myers *et al.* 1995), as advised by J. Ianelli (pers. comm).

Estimable parameters:

The sector-specific fishing selectivities S_a^s are assumed to follow the functional form:

$$S_a^s = \frac{P \cdot e^{-\mu a}}{1 + e^{-\delta(a - \tilde{a})}} \tag{A22}$$

where μ, δ and $\tilde{a}$ are three estimable parameters that control the shape of the function and P is simply a scalar fixed at a value such that $S_{11}^s = 1.00$. In essence, μ controls the slope of the right hand limb of the function, δ controls the steepness of the ascending left hand limb, and $\tilde{a}$ shifts the function to the left or right, all in relation to age a.

THE LIKELIHOOD FUNCTION

The likelihood function which is maximised in the parameter estimation process is based on equations developed by Geromont & Butterworth (1999). The model is fitted to abundance and catch-at-age data and the contributions by each of these to the negative of the log-likelihood ($-\ln L$) calculated as described below.

Abundance data:

The likelihood contribution is calculated assuming that the observed abundance index is log-normally distributed about its expected value:

$$I_y^s = \hat{I}_y^s e^{\varepsilon_y^s} \qquad \text{or} \qquad \varepsilon_y^s = \ln(I_y^s) - \ln(\hat{I}_y^s) \tag{A23}$$

where I_y^s is the abundance index for year y and sector s, $\hat{I}_y^s = q^s B_y^{exp,s}$ is the corresponding model estimated value, where $B_y^{exp,s}$ is the model value for exploitable resource biomass corresponding to sector s, given by equation (10) (if the index refers to numbers, $B_y^{exp,s}$ is replaced by $N_y^{exp,s}$–see equation (11)). q^s is the constant of proportionality for abundance series corresponding to sector s, and ε_y^s from $N(0,(\sigma^s)^2)$.

The contribution of the abundance data to the negative of the log-likelihood function (after removal of constants) is given then by:

$$-\ln L = \sum_{s}\left[\sum_{y} \ln \sigma_{s}^{y} + (\varepsilon_{s}^{y})^{2}/2(\sigma_{s}^{y})^{2}\right] \qquad \text{(A24)}$$

Variance unspecified: (CPUE abundance series)

In this case the standard deviation of the residuals for the logarithms of abundance series s is assumed to be independent of y, and is estimated in the fitting procedure by its maximum likelihood value:

$$\hat{\sigma}^{s} = \sqrt{\frac{1}{n_{s}}\sum_{y}(\ln I_{s}^{y} - \ln \hat{I}_{s}^{y})^{2}} \qquad \text{(A25)}$$

where n_s is the number of data points for the abundance series corresponding to sector s.

The catchability coefficient q^s for sector s's abundance index is estimated by its maximum likelihood value:

$$\ln \hat{q}^{s} = \frac{1}{n_{s}}\sum_{y}(\ln I_{s}^{y} - \ln \hat{B}_{s}^{\text{EXP}\,y}) \qquad \text{(A26)}$$

Variance specified: (FIAS data)

The catchability coefficient q^s for this sector's abundance index is estimated by its maximum likelihood value which, for the case of a log-normal error distribution is given by:

$$\ln \hat{q}^{s} = \frac{\sum_{y} 1/(\sigma_{s}^{y})^{2}(\ln I_{s}^{y} - \ln \hat{B}_{s}^{\text{EXP}\,y})}{\sum_{y} 1/(\sigma_{s}^{y})^{2}} \qquad \text{(A27)}$$

where $(\sigma_s^y)^2 = \ln(1 + (CV_s^y)^2)$ and the coefficient of variation (CV_s^y) of the resource abundance estimate for year y is input.

Catches-at-age:

The likelihood contribution is calculated assuming a log-normal error distribution and by making an adjustment (suggested by A. Punt, pers. comm.) to weight in inverse relation to sample size so that undue importance is not attached to data based upon a few samples only:

$$-\ln L = \sum_{y}\sum_{s}\sum_{a}\left[\ln\left(\sigma_{c}^{s}\sqrt{\hat{p}_{s,a}^{y}}\right) + \hat{p}_{s,a}^{y}\right.$$
$$\left.\left(\ln p_{s,a}^{y} - \ln \hat{p}_{s,a}^{y}\right)^{2}/2\left(\sigma_{c}^{s}\right)^{2}\right] \qquad \text{(A28)}$$

where $p_{s,a}^{y} = C_{s,a}^{y}/\sum_{a} C_{s,a}^{y}$ is the observed proportion of abalone caught/sampled by sector s in year y that are of age a, $\hat{p}_{s,a}^{y} = \hat{C}_{s,a}^{y}/\sum_{a} \hat{C}_{s,a}^{y}$ is the model-predicted proportion of abalone caught/sampled by sector s in year y that are of age a, where:

$$\hat{C}_{s,a}^{y} = N_{s,a}e^{-M/4}S_{a}^{s}F_{s}^{y} \qquad \text{A29}$$

and σ_c^s is the standard deviation associated with the catch-at-age data for sector s, estimated in the fitting procedure by:

$$\sigma_{c}^{s} = \sqrt{\sum_{y}\sum_{a}\hat{p}_{s,a}^{y}(\ln p_{s,a}^{y} - \ln \hat{p}_{s,a}^{y})^{2}/\sum_{y}\sum_{a}1} \qquad \text{(A30)}$$

Inspection of the various $-\ln L$ contributions revealed that the catch-at-age $-\ln L$ contributions were substantially larger than those for CPUE and the FIAS series, in part because they include many more data points because of summation over age as well as year. This is questionable as the $p_{s,a}^{y}$ values for a given y and s are not likely to be independent (as implicitly assumed by equation (28)), because the cohort-slicing method used to provide the catch-at-age information from length composition data likely introduces positive correlation. The catch-at-age $-\ln L$ contributions were thus downweighted by a multiplicative factor of 0.1, thereby downscaling these contributions to a similar order of magnitude as the CPUE and FIAS contributions.

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SUSTAINABILITY DEMANDS VIGILANCE: EVIDENCE FOR SERIAL DECLINE OF THE GREENLIP ABALONE FISHERY AND A REVIEW OF MANAGEMENT

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Who hears the fishes when they cry? It will not be forgotten by some memory that we were contemporaries.

Henry David Thoreau

ABSTRACT The greenlip (*Haliotis laevigata*) abalone fishery in the Western and Central Zones of South Australia comprises about 74 distinct metapopulations. Although the total catch has been maintained since 1979, analysis of catch and effort data for the two zones at a fine scale in 49 map code areas shows that 39 areas are in decline; of these, 28 areas, with declines exceeding 50%, are considered serious. In the Western Zone, the proportional catch from 23 inshore reefs has declined from ~69 to ~34% of the total catch over 20 y of fishing; whereas, that of 10 distant reefs has increased from ~26 to ~50% of the total over the same period. Fishing mortality is 0.4–0.6 on inshore reefs and 0.2–0.4 on distant ones, indicating serious maldistribution of effort. The frequency of declining populations reaching the 50% decline point peaked in the years 1989 to 1990; a subset of the same group of populations reaching the 80% decline point peaked in 1995 to 1996, indicating a continuing decline trajectory, despite quota imposition and reduction. Multiple regression analysis of the proportional rate of decline of 38 populations in the Western Zone showed that the rate of decline was inversely correlated with distance from port and with a closure index that measures the extent to which the population is bounded by land. The closure index is considered to estimate crudely the extent to which coastal topography retains larvae within the population. Comparison of the spatial extent of fishing areas in eight populations mapped in 1978 to 1979 and again in 1996 to 1999 showed strong spatial contraction matching the decline in catch. Spatial contraction occurred from deeper to shallow water leaving relict subpopulations around headlands and in bays, consistent with inferences on the effect of coastal topography on abalone larval retention. In the Central Zone, with six divers and 12 reefs fully fished over a shorter time period, the declines are less certain and less severe. The catches of five reefs have declined by >50% and increased substantially on one remaining productive reef. An overfishing hypothesis best explains the long-term decline in the catch of many reefs and the transfer of fishing to distant or remaining reefs.

The history of management is reviewed and shows that, over the lifetime of the fishery, management has moved from control by government with little consultation with industry in 1967 toward self-management with endorsement by government from about 1995. Since 1995, management has been unresponsive to declines in the fishery, and possible reasons are explored. We advocate application of the precautionary approach by management at the scale of the metapopulation to arrest the declines and the establishment of recovery plans for areas in serious decline.

KEY WORDS: abalone fishery, serial decline, *Haliotis laevigata*, metapopulation, larval retention, fishery indicator, precautionary approach, fishery management

INTRODUCTION

Focus on the causes and prevention of collapse of fisheries is a direct consequence of increasing emphasis on the conservation of exploited stocks and their sustainable management. The spectacular collapses of anchovetta, cod, and crustacean stocks in recent decades (Steele 1981, Hutchings & Myers 1994, Orensanz et al. 1998) have served to catalyze deep concern and critical analysis of the causes in the hope that appropriate lessons can be learned.

Many abalone fisheries have also collapsed worldwide (Shepherd & Baker 1998), except in Australia, where they have apparently been sustained (reviewed in Prince & Shepherd 1992), and where envious eyes have often turned to learn the touchstone of success. The collapse of abalone fisheries has many causes underlying the obvious proximate cause of overfishing. Basic problems of assessment are: ignorance of stock–recruitment relations and practical difficulties and high cost of obtaining fisher-independent data, such as abundance and recruitment estimates for many independent stocks. Problems confronting managers are: absence of proved fishery indicators and tendencies to maldistribution of effort. Yet, despite their reputation, all is not well with the Australian abalone fisheries. In particular, the fishery for greenlip abalone (*Haliotis laevigata* Donovan) has suffered declines of varying severity in several states (Shepherd et al. 2001), despite the fact that possibly more is known of the biology and population ecology of this species than any other abalone species. This paper seeks to discover an underlying pattern in the declines and why they are continuing in South Australia, despite the abundant knowledge and the recent warnings of decline. We focus our attention on the greenlip fishery, although blacklip abalone, *H. rubra* Leach, is taken on most reefs, although in different habitat.

First, we summarize the diverse evidence, both biologic and historic, showing a decline in the fishery, and undertake a preliminary analysis of factors associated with that decline. The recent (1995) availability of catch data at a fine (metapopulation) scale (Keesing & Baker 1998) has provided a catch history of some 49 independent greenlip stocks in the Western and Central Zones (see Shepherd & Brown 1993); our analysis is limited to these zones. We consider the evolution of management from government control toward self-management in the context of the changing legal and administrative structures. We then describe the history of key management decisions and explain the long-term declines in terms of failure to apply a precautionary approach. We conclude with recommendations for the improvement of management and the

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restoration of the stocks. Our conclusions are relevant generally to management of exploited, sedentary invertebrates with multiple stocks.

METHODS AND MATERIALS

Historical Information and Catch and Effort Data

Historical data were extracted from the minutes of the Abalone Advisory Committee and later of the Abalone Management Committee (AMC) and the records of the first author, who has been involved in research and management of the fishery continuously since 1968. Catch and effort data, reported on monthly logbooks that recorded daily fishing excursions, have been collected since the inception of the fishery in 1967. Early data on catch and hours fishing were collected on a grid scale of ~37 × 37 km, and, from 1979, on a fine population scale with map code numbers (Fig. 1) allotted to individual reefs, islands, and sections of coast, reflecting the actual distribution of abalone populations. Thus, the Western and Central Zones have 153 map codes embracing about 164 distinct abalone metapopulations. Excluding the far west region north of Pt. Brown, which has ~34 greenlip populations, there are 138 reef codes embracing ~74 greenlip and a similar number of blacklip metapopulations. The number is approximate, because we have assumed that isolated reefs and islands have distinct metapopulations and that mainland populations separated by unsuitable habitat of >~20 km are distinct (see Shepherd & Brown 1993). The term population in this paper (names are followed by map code numbers in brackets for easy cross reference) means an inferred metapopulation, or in some cases, two or more juxtaposed metapopulations, following the detailed genetic work of Brown and Murray (1992). The populations considered and their corresponding map codes are listed in Tables 1, 2. Our study is confined to 49 map code areas, including Franklin I. but excluding the seldom visited, very small populations at Greenly I., west Spencer Gulf and Sir Joseph Banks Group in the Western Zone, and Cowell Grounds and Marion and Stansbury Reefs in the Central Zone with a total mean cumulative annual catch of ~1 t.

Catch and effort data at the scales described are given by Keesing et al. (2000), with partial analyses of specific features by Keesing and Baker (1998) and Shepherd and Baker (1998). Pending a full analysis of the data, we have here undertaken a preliminary analysis to identify important features deserving detailed study. We estimated rates of decline in the catch of individual populations by simple regression analysis, over the full time period (usually 19 or 20 y from the commencement of intense fishing) where the decline was monotonic, or with a broken stick model, where the catch fell sharply and then stabilized at a low level; in the latter case, we estimated the rate of decline by regression from the start of fishing to the year in which the catch had declined by 95%. For a few areas, mostly in the Central Zone, catches were initially low and peaked during the mid- to late-1980s; in these cases, the regressions to estimate rates of decline commenced at the point where catches first peaked, our object being to estimate decline rates from the time of peak catches rather than in relation

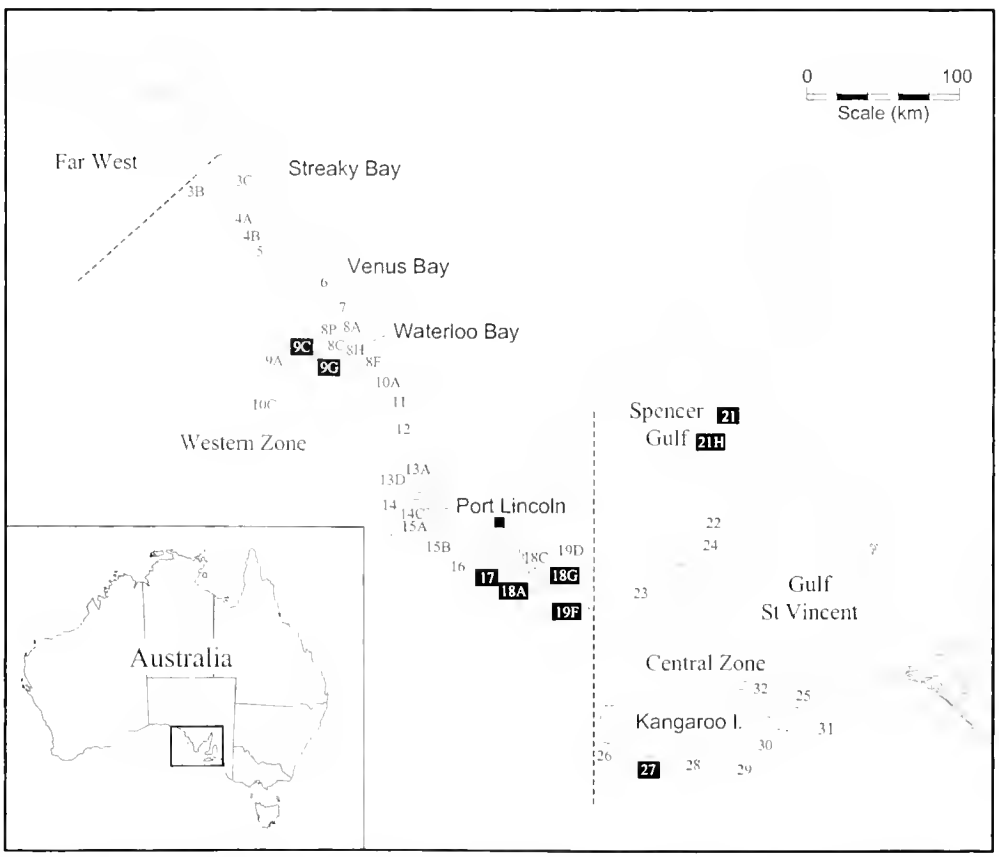

Figure 1. Map of South Australia showing distribution of greenlip abalone populations, individually indicated by map code numbers, in the Western and Central Zones. Numbers within black rectangles are the map codes of populations whose catches have been stable or have increased over time. See Tables 1 and 2 for names of numbered reefs.

TABLE 1.

Greenlip abalone populations showing declines in the catch in the Western Zone (Map codes 3–19) and Central Zone (Map codes 22–32).

Map Code	Name	Historic Catch (t) (1971–1978)	Early Catch (t)	Current Catch (t) (1996–1998)	Rate of Decline	% Down	Z or D
3B	Franklin Is.	1.7	6.5 (84)	4.3	0.62 (11 y)	70	
3C	Cape Bauer	0.7	6.7 (84)	0.8	0.46* (15 y)	91	
4A–C	Highcliff	9.5	11.9 (80)	4.3	0.49***	63	
4D,E	Sceale Bay	2.8	5.3 (79)	0.6	0.27**	98	0.60
4F–H	Cape. Blanche	5.6	8.6 (80)	3.7	0.23 NS	45	
5A	Pt Labatt	2.8	4.7 (80)	2.8	0.21 (13 y)	50	
5B–F	Baird Bay	11.2	21.0 (82)	13.9	0.33 NS	39	
6A–D	Venus Bay	2.1	9.2 (82)	6.7	0.22 NS (16 y)	38	
6E,7A,B	Anxious Bay	9.5	7.3 (80)	1.0	1.4*** (10 y)	85	<0.01/m^2
8C,D	Watchers	7.5	9.0 (80)	1.1	0.52** (19 y)	91	
8A,E,Q	Waldegrave I	25.9	21.2 (80)	17.4	0.80**	56	0.61
8H–M	Waterloo Bay	28.5	12.5 (79)	0[a]	3.3*** (11 y)	>95	0.8
8F,G,N	Elliston Cliffs	2.4	3.1 (80)	0.3	0.20*** (19 y)	99	
8P,9E,F	Flinders I (Gem)	15.7	16.4 (80)	11.2	0.35 NS	41	
9A,B	Ward I	13.6	13.5 (80)	13.1	0.07 NS (19 y)	12	0.45
10A,B	Tungketta	0.3	2.6 (83)	0.1	0.36* (10 y)	>99	
10C	Pearson I.	0.7	1.0 (82)	0.3	0.12** (17 y)	97	0.25
11A	Sheringa	4.1	3.9 (80)	3.1	0.13*	63	
11B,C	Kiana	3.4	4.2 (80)	0.5	0.25*** (17 y)	>99	
12A,C	Drummond	20.6	23.7 (80)	2.6	1.53*** (17 y)	>95	0.59
13A,C	Frenchman	3.7	5.3 (80)	0.3	0.24***	98	<0.01/m^2
13D–F	Reef Head	13.9	13.2 (80)	2.9	2.09** (14 y)	>89	
14A,B	Pt. Whidby	4.4	4.7 (80)	0.9	0.49** (14 y)	>95	
14C,D	Misery Bay	27.8	30.6 (80)	7.8	1.58**	95	
14E	Whidby Is.	0	13.7 (83)[b]	1.2	0.30 NS (11 y)	91	
15A	Avoid Bay	49.1	47.5 (80)	2.2	3.17***	>95	0.60
15B,16A	D'Anville Bay	5.8	3.8 (80)	1.6	0.15**	75	
16C	Fishery Bay	18.3	12.8 (80)	4.4	0.48***	79	
18C–F	Thorny Passage	40.0	44.2 (80)	37.5	0.30 NS	13	1.0
19A–C							
19D,E	Dangerous Reef	0.4	2.4 (83)	0.8	0.10 NS (16 y)	49	
22A,24A	Hardwicke Bay	2.7	29.7 (84)[c]	12.4	4.0** (12 y)	87	
23	SW Yorke	5.3	4.6 (80)	1.8	0.17 NS (10 y)	48	
25A	Backstairs	6.2	6.6 (80)	0.5	0.40 NS (15 y)	76	0.41
26	West Bay	1.0	4.0 (85)	2.2	0.27 NS	29	
28	Vivonne Bay	1.8	2.2 (82)	0.7	0.20** (17 y)	98	
29	Gantheaume	4.3	3.9 (80)	3.4	0.09 NS	37	
30	D'Estree Bay	4.5	4.8 (82)	2.5	0.20 NS (17 y)	42	
31	Willoughby	3.7	3.6 (82)	2.5	0.59* (10 y)	90	
32	NE Kangaroo I	2.0	2.0	2.0	0.14 NS (10 y)	50	

Historic catch is the mean annual catch (t) for 1971 to 1978, early catch is the mean annual catch for 3 y commencing with the year shown in brackets, current catch is the mean annual catch (t) for 1996–1998, rate of decline (t/yr^{-1}) is for the period 1979 to 1998 or a lesser period where indicated, percentage decline is the present productivity as a percentage of original production (see text), Z is the instantaneous total mortality rate from catch curve analyses, and survey densities, D, are in numbers/m^2. A blank indicates absence of data. Statistical significance is shown thus: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; NS = not significant. [a]closed to fishing 1982–1986 and 1995–1999. [b]reef discovered 1983. [c]reef discovered 1984.

to any specific fixed starting point. The 50 and 80% points of decline and total percentage declines were determined from the regressions. The mean annual catch for 1971 to 1978 (Table 1) is given to provide an estimate of productivity early in the fishery after removal of the virgin stock from 1967 to 1970. The "early catch" mean annual catch data in Table 1 are the mean annual catches over 3 y in the early 1980s after stabilization of the catches following granting of the right of divers to sell their licences in 1980. The mean annual catches were then only very slightly lower (<0.2%) than the mean annual catches for 1971 to 1978 and only 12.6% higher than the mean annual catch for the first 4 y after quota introduction in 1985. The current catch data given (mean annual catch for 1996 to 1998) are estimates of the present productivities of populations. In this paper, productivity is estimated by the annual catch of a stable population averaged over a number of years and assumed to be more or less fully exploited (Kesteven 1996).

All catch data are given in total (in-shell) weights (TW). Estimates of the total mortality coefficient, Z, are from: Shepherd and Baker (1998) for Waterloo Bay (8H–M), Sceale Bay (4D,E), Ward I. (9A,B), Thorny Passage (18C–F) and Tiparra (21); Shepherd et al. (2001), for Avoid Bay (15A) and Backstairs Passage (25A); and O'Loughlin and Shepherd (unpublished data) for the remaining sites. Mean density estimates are unpublished data.

TABLE 2.

Greenlip abalone populations in the Western and Central Zones with stable or increasing catches.

Map Code	Name	Historic Catch (t) (1971–1978)	Early Catch (t)	Current Catch (t) (1996–1998)	Rate of Change	% Up	Z or D
9C,D	Hotspot	23.0	26.2 (79)	26.1	+0.01 ns	1.3	0.30
9G,H	Flinders SE	7.1	8.3 (80)	11.1	+0.13 ns	40	
17A,B	Sleaford	2.4	1.4 (80)	2.4	+0.02 ns	26	
18A,B	West Pt.	8.8	5.3 (79)	6.3	+0.17 ns	23	
18G–L	Thistle I	8.5	9.8 (80)	8.4	+0.008ns	3	
19F	Wedge I.	2.9	3.7 (80)	5.3	+0.08 ns	88	
21	Tiparra	45.1	67.8	96.4	+2.16 ns	77	0.72
21H	C. Elizabeth	7.4	25.4 (79)	11.7	+0.03	10	
27A	Hanson Bay	1.1	0.5 (79)	2.2	+0.04	87	

See caption to Table 1 for explanation of headings.

Estimates of the spatial extent of fishing areas in 1978 to 1979 and in 1995 to 1999 were made during individual diver interviews by one of us (SAS) when divers marked on maps at a scale of 1 km = 1 cm (twice that scale for Watchers (8C,D) and Fishery Bay (16C)) the spatial extent of the places they fished. Hence, the data show only the gross extent of greenlip habitat, including interspersed sandy patches, rather than the precise extent of rocky substratum actually occupied by abalone. The areas shown here were drawn from the common area indicated by a minimum of four divers in 1978 to 1979 but only three divers in 1995 to 1999, because fewer divers fished areas that contracted spatially.

In the multiple regression analysis, the dependent variable, the proportional rate of decline of catches for individual reefs, was the absolute rate of decline calculated as described above divided by the initial production that was the y-intercept of the regression of catch versus time. The independent variables examined were: initial production (as defined above), distance from port, depth, and a "Closure Index" of the population. Distance from port was the distance from the mid-part of the reef in question to the nearest mainland boat ramp along the sea route used by divers; depth was the average depth of the population as then exploited as supplied by divers during the 1978 to 1979 diver interviews; and the "Closure Index" was the proportion of the perimeter of the areal extent of the fished reef bordered by land or emergent reef. For this purpose, reef maps prepared from the diver interviews described above were used to calculate the index, which took a value between 0 and 1.

History of Management

The history of the fishery has been reviewed by Prince and Shepherd (1992) with later detail added by Keesing and Baker (1998). Here, we summarize briefly the salient features of fishing controls and management. The fishery is controlled by input measures, size limits, and licence limitation, and by output measures, and individual quotas.

After the virgin stocks were fished down by 1970 (Shepherd & Baker 1998, Keesing et al. 2000) and the number of divers reduced to 21 in the Western Zone and 5 in the Central Zone by 1971, the fishery was stable for the next 9 y. In 1973 to 1975 there was slight evidence of stagnation in the fishery as divers aged until three more divers were admitted in 1976. In 1980, licences became saleable, and catches increased somewhat until 1984, when growth overfishing became marked in the Western Zone and various controls were introduced to reduce the level of fishing (see below). In 1985, individual quotas were introduced in the Western Zone (12 t for greenlip) and in 1989 in the Central Zone (23.7 t for greenlip). Greenlip quotas were reduced by 3 t (25%) in the Western Zone in 1989. During the 1990s, as inshore catches progressively declined in the Western Zone, competitive behavior between divers became evident as divers at the beginning of the fishing year competed to be the first to fish the fishing grounds and maximize their catch rates.

Management has evolved progressively since 1967. From 1967 to 1978 the Director of Fisheries managed the fishery, under delegation from the Minister of Fisheries. In practice, decisions were made on scientific advice and/or in response to representations by the divers' association. Such key decisions as licence limitation and their early reduction in number, and the imposition of zones in the fishery were made in accordance with ministerial policy and sometimes in conflict with the wishes of divers. This may be called the "instructive" period of government management (Fig. 2) after the terminology of Sen and Raakjaer Nielsen (1996). In 1978, following a prolonged dispute in which divers resisted the addition of more licences to the fishery (Chatterton & Chatterton 1981), an advisory committee was established, comprising divers and government representatives in about equal numbers. The committee had no authority, but made recommendations on research and management. Matters, some of them contentious, considered by the committee in the first 7 y included: the issue of rewards by indus-

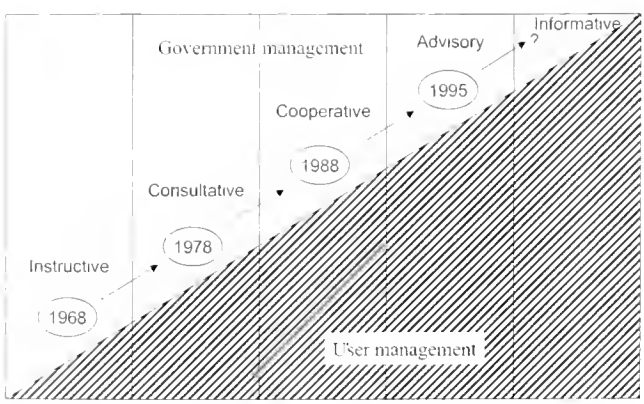

Figure 2. Shift in comanagement arrangements over time in the South Australian abalone fishery. The years in brackets indicate approximately the suggested point of transition along the spectrum of change.

try for evidence of poaching; relief diver days (awarded during sickness of licensee); landing abalone in the shell; permit sharing; research closures; increase in size limits; and a buy-back scheme. On major issues, such as the admission of more divers and the right to sell licences, divers dealt directly with the government and bypassed the committee, but on other issues, divers' views were taken into account in the final decision. During this period, the arrangement could be termed *consultative* in terms of mode of influence (Hersoug & Ranes 1996) for major issues and *cooperative* for minor issues (Fig. 2). Thus, major issues were resolved by the Director or Minister and minor ones in a cooperative manner within the committee.

By 1985, divers were showing increased responsibility in terms of resource conservation and management following evidence of overfishing in 1981–1983 (Lewis et al. 1984). Divers urged a 4-mo closure of the Western zone during 1984 to reduce the catch from the overfished region, and agreed to an increase in size limit from 130 to 145 mm SL for that zone (advocated by researchers since 1979). They also proposed closures during the spawning season, increased the reward to $5,000 for information about poaching, investigated the feasibility of quotas, and then voluntarily assumed quota restrictions before promulgation of the amending regulations, assented to the abolition of relief diver days, and offered assistance with research.

In 1988, an abalone management committee (AMC) was formally established to "advise on management, harvesting strategies, and on policy and legislation." Divers instigated the landing of abalone in the shell in the Central and southern Zones (later modified), and the introduction of quotas in those zones in 1988 and 1989.

In 1989, the greenlip quota in the Western Zone (see Fig. 6) was reduced by 3 t TW (25%) upon evidence of declines in inshore populations and in partial recompense for the loss, an increase in the blacklip quota of 0.75 t TW was negotiated by the divers from 1990. A decline in the catch in Backstairs Passage was reported by research in 1989, and an increase in size limit for greenlip on Kangaroo Island was proposed, but not accepted. Thus, during the 1980s, divers took the initiative and were pro-active on many, but not all, measures directed toward the conservation of the stocks.

In 1989, the president of the Abalone Divers Association was appointed chairperson of the management committee in tacit acknowledgment of the industry's constructive participation in management. From this time, it might be claimed that management was joint with government, although formal acknowledgment of the *de facto* joint management arrangements did not occur until 1995, when the requisite regulations establishing the AMC became law. By 1992, the right of permit holders to employ divers to take their catch was recognized, and the practice began and soon became widespread, for licences to pass into the control of companies, in which one or more divers, under contractual or share fishing arrangements, were nominated to take abalone. The implication of this was that, as time went on, divers' representatives on the management committee became increasingly likely to be business persons with little first-hand knowledge of abalone abundances in the field.

The 1995 regulations essentially passed control of the fishery to divers, because only industry members had voting rights. Four government observers on the committee represented administration, management, research, and enforcement sections of government; subsequently other observers representing the umbrella association of all professional fishers, recreational, and conservation interests were added. The Director of Fisheries retained the power to set quotas, but in practice, quotas were recommended by the government manager, endorsed by the AMC, and approved by the director. Under the regulations, the Minister reserved the power to override decisions of the AMC, but this has not happened. By 1995, government policy was explicitly one of cost-recovery; i.e., the industry was required to pay all costs of administration, management, enforcement, and research in the fishery. In consequence, the AMC increasingly involved itself in the scrutiny of government expenditure on research and management and "demanded the types and levels of service that offered the greatest value for money" (Geen & Nayar 1988).

In 1997, an independent chairman of the management committee was appointed, in accordance with government policy to have chairpersons with no financial interest in the fishery. Thus, from 1995, responsibility and power have increasingly passed to industry, and government observers have increasingly been limited to an advisory role. Overall, as shown in Figure 2, a transition from government management with little consultation, toward self-management with endorsement by government has occurred from 1967 to the present. At the same time, we may observe an increasing acceptance of responsibility by divers who, during the 1980s up till 1995, proposed many of the measures actually adopted to conserve stocks.

In 1995, the AMC was first advised of declines in eight greenlip stocks (a subset of those in Table 1) and a few blacklip ones in the Western Zone, and workshops were held in 1995, 1996, and 1999 with industry to consider them. Researchers advocated closures and an increase in size limits, but these were resisted, and no action resulted. Annual stock assessments and other reports to the AMC from 1995 to 1999 reiterated the serious character of the declines and assessed the impact of a size increase. In 1999, industry accepted that there were problems in the fishery, and a stock recovery plan was initiated for four sites: Avoid Bay (15A), Frenchman (13B,C), Anxious Bay (6E,7A,B), and Sceale Bay (4D,E). Unfished, stunted abalone subpopulations occurred near these sites, and researchers and industry cooperated in the translocation of adults into the fishing grounds to increase larval settlement.

RESULTS

Catch Declines

The Western Zone is subdivided into two subzones, A and B. Subzone B (the far west) has low productivity, and the annual quota is correspondingly low (1.8 t per diver unallocated between blacklip and greenlip); greenlip catches have fluctuated cyclically but have shown no long-term downward trend. We do not consider them further in this study.

The total catch for the two zones (Fig. 3) has remained stable save for a slight reduction in catch in the Western Zone with the 1989 quota reduction. The zones comprise many spatially separated greenlip populations of different productivities geographically distributed on exposed coasts and near the gulf entrances, except for Tiparra (21) and Hardwicke (22,24) within Spencer Gulf (Fig. 1). The size distribution of these populations, in terms of their 1971 to 1978 mean annual productivity values (see Table 1) for each zone (Fig. 4) shows that the majority of reefs had productivities of <10 t and that the majority in each productivity size class in each zone have suffered declining catches since 1979. Examples of the declines in catch in individual populations are

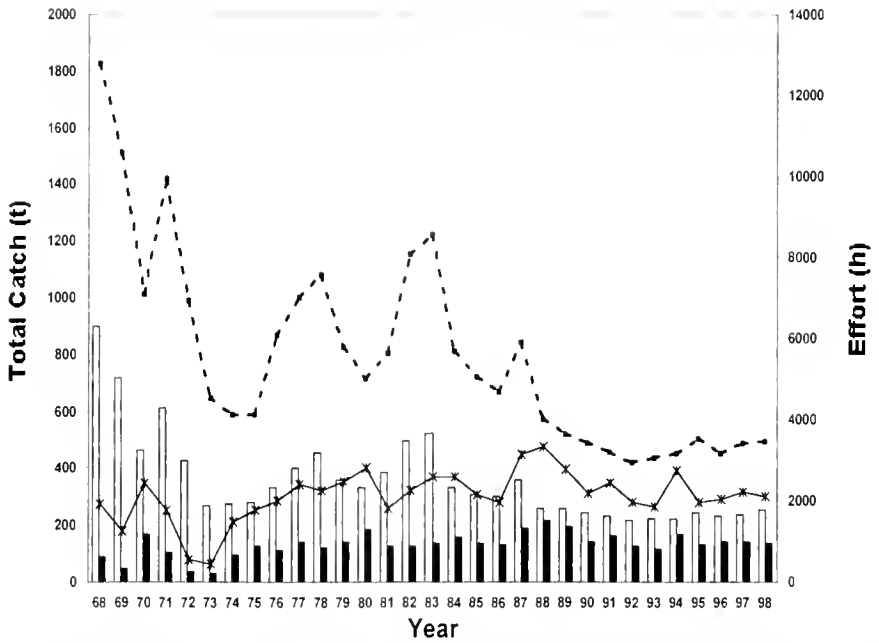

Figure 3. Global greenlip catch (TW) and effort in the Western and Central Zones from 1968 to 1998. Data are for financial years, July to June; i.e., 68 = 1968 to 1969. Hollow bars (total catch) and dotted line (total effort) are for the Western Zone and black bars and continuous line are for the Central Zone.

given in the companion paper (Shepherd et al. 2001) and in Shepherd and Baker (1998).

Historic and present catches of declining populations, rates of decline, and the percentage decline (derived from regression analyses), and estimates of the total mortality coefficient, Z, and recent density estimates are summarized in Table 1. Historic and early annual catch data (Table 1) are taken to indicate the approximate productivity of reefs over a decade or more during the 1970s and early 1980s, and current annual catch data are taken to indicate the present productivities of those reefs. In all, 39 reefs show declines in catch of 12 to >99% (mean % decline 71%) in the two zones (Table 1) and six reefs show no decline or increasing catches (Table 2). These percentage decline values represent the declines from maximum catches in 1980 to 1982, equivalent to the mean annual catches during the 1970s after removal of the virgin stocks.

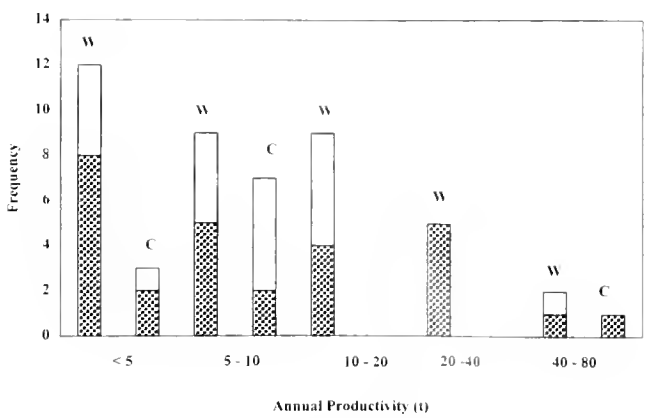

Figure 4. Frequency distribution of the annual productivity (t) estimated from the mean catch of greenlip-bearing reefs in 1971 to 1978 in the Western (W) and Central (C) Zones. Reefs whose catches have declined by >50% are shown by crosshatching.

Next, we examined the temporal pattern of the decline in catch in the Western Zone by plotting the frequency distribution over time of populations reaching their respective 50 and 80% decline points after 1980. The results (Fig. 5) show that the number of declining reefs (extracted from the data in Table 1) at the 50% decline point peaked in 1989 to 1990, and the number at the 80% decline point (a subset of the same reefs) peaked from 1995 to 1996.

Changes in Fishing Pattern

The pattern of fishing gradually shifted from 1979 to 1998 in both zones. In the Western Zone, as near-shore catches declined, fishers increasingly sought their catch from more distant reefs. This long-term shift in the source of the catch is shown by the gradual proportional decline of the catch in 23 near-shore populations from ~65 to 70% in 1979 to ~34% in 1998, and the corresponding proportional increase in catch at 10 distant reefs from ~26% to ~50% over the same period (Fig. 6). Distant reefs are 30–50 km from the nearest port, except Venus (6A–D), Baird Bay (5B–F), (which are close to port but distant from Port Lincoln where most fishers live) and Sleaford (17A,B). The remaining reefs cumulatively contributed about the same proportion to the total catch over time. In the Central Zone, the picture is simpler. As catches declined on nine reefs around Kangaroo I. and Hardwicke Bay (22,24) (Table 1) they progressively increased at one reef, Tiparra (21), by a massive 77%.

To determine whether the spatial shift in fishing in the Western Zone was related to more intense fishing on near reefs than on distant ones, we examined Z for 11 reefs (see Tables 1,2). A plot of Z versus distance (D) from port (Fig. 7), shows a decline in Z with increasing distance. We fitted the linear regression equation:

$$Z = 0.71 - 0.008D \qquad (R^2 = 0.56;\ P < 0.01)$$

For this regression, we used the earliest Z estimates available for Avoid Bay (1987) and Backstairs Passage (1980); whereas, the

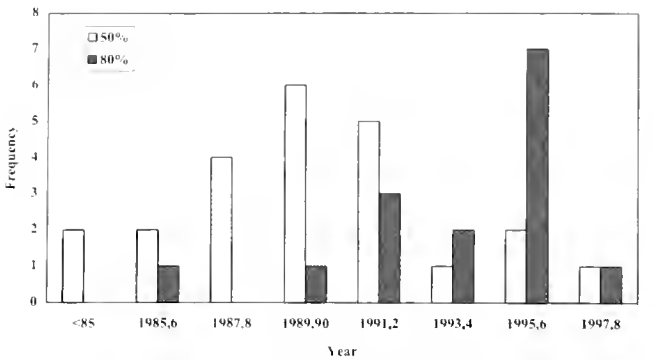

Figure 5. Distribution over time of number of greenlip populations in the Western Zone that reached 50% (blank) and 80% (striped) decline points during their decline trajectory.

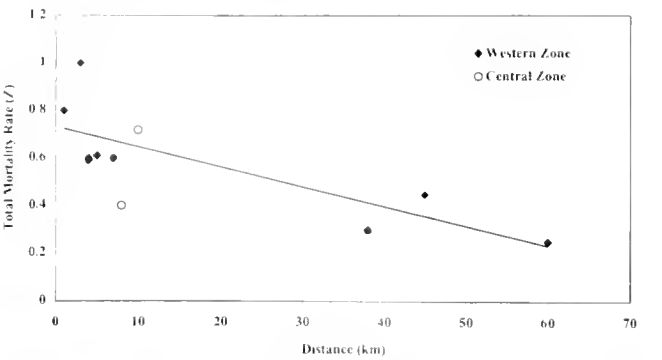

Figure 7. Plot of total mortality coefficient, Z, for greenlip populations in the Western and Central Zones versus distance from port in km.

remaining estimates were obtained since 1995 (Shepherd & Baker 1998, O'Loughlin & Shepherd, unpublished data).

Rate of Decline in Catch

The relation between the proportional *rate* of decline of the catch in declining populations, and the variables: initial production (IP), distance (D), depth (De), and the "Closure index" (C) for populations in the Western Zone was examined by multiple regression analysis. The constant and the two variables, D ($P = 0.002$) and C ($P = 0.01$) were significant, but the variables IP and De were not significant and were removed from the regression. The regression equation (with standard errors in brackets) is:

$$R = 9.4(1.6) - 0.07(0.02)D - 9.2(3.5)C$$
$$(R^2 = 0.31;\ P = 0.0006)$$

Plots of the proportional rate of decline of reefs versus the variables IP, D, and C, but not De (which shows little of interest), are shown in Figure 8.

Spatial Contraction of Fishing Areas

Plots of the recorded spatial contraction of fished reefs in six greenlip populations are shown in Figure 9. The respective spatial extent of areas originally and presently fished are given in Table 3, with the calculated percentage contraction of fishing areas. Table 3 also includes the relevant data for the two populations, Avoid Bay (15A) and Backstairs Passage (25A), whose declines are described in greater detail by Shepherd et al. (2001). The spatial declines have been groundtruthed for four of the eight populations (see Table 1 and Shepherd et al. 2001), and they show very low densities in the areas of decline. Such low abundances are not surprising, because divers fish the areas in motorized scooters (Prince & Shepherd 1992) and cover the bottom systematically and over distances of >10 km per dive. The low relief, calcrete (limestone) substratum, sometimes interspersed with sandy patches (see

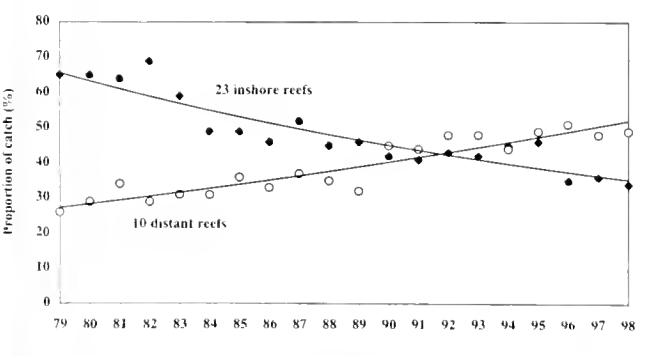

Figure 6. Proportional change in of the greenlip catch for twenty-three near-shore and ten distant reefs in the Western Zone from 1979 to 1998. The distant reefs are Venus Bay, Ward I., Hotspot, Flinders I. (both sides), Baird Bay, Sleaford, Thistle I., Wedge I., and southern Thorny Passage, and the near-shore reefs are the remainder of those listed in Table 1, other than Central Zone reefs.

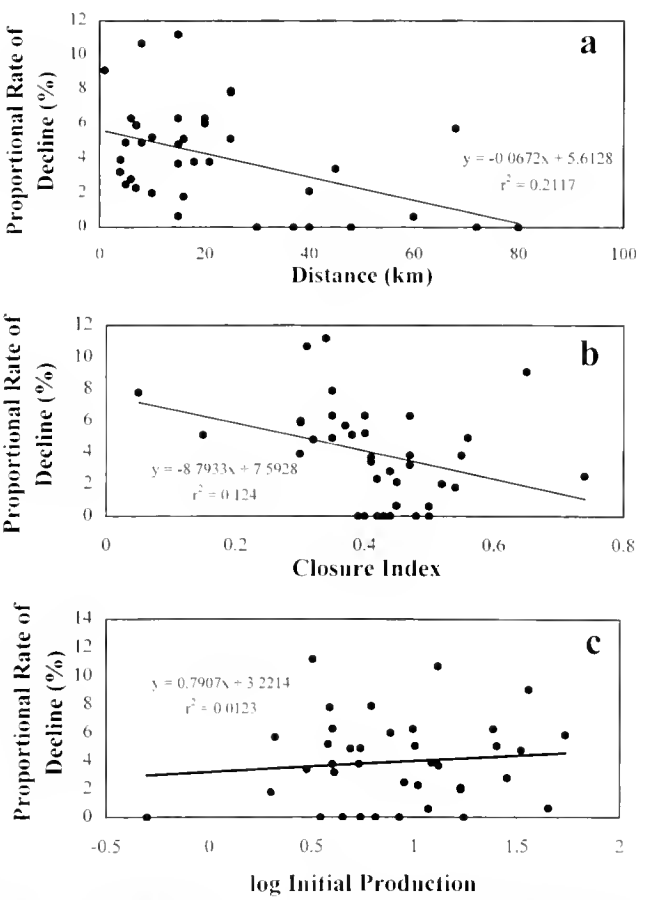

Figure 8. Graphs of the proportional rate of decline of greenlip populations in the Western Zone versus a: distance; b: closure index; and c: log (initial production).

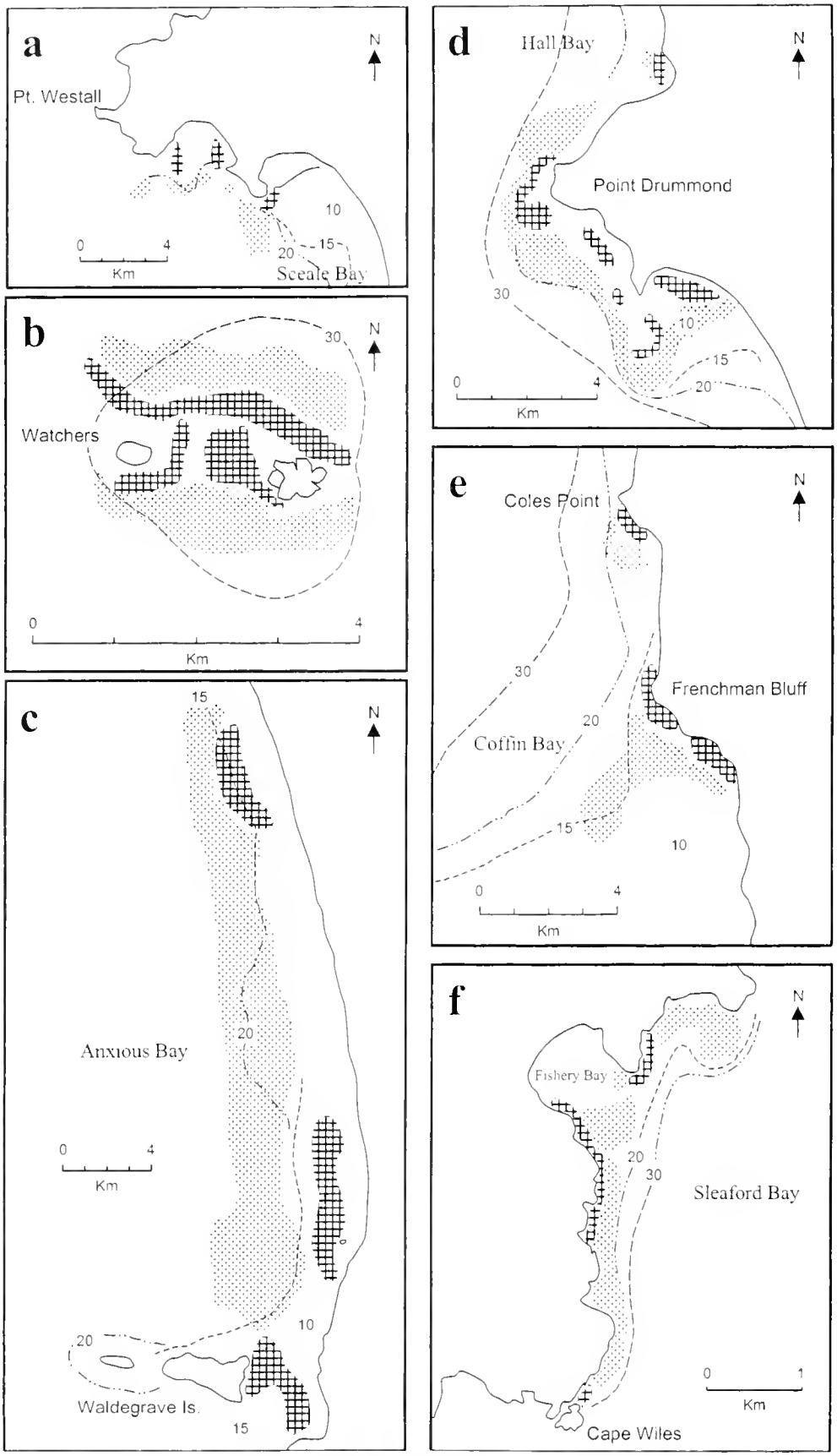

Figure 9. Spatial contraction of six greenlip populations in the Western Zone. a: Sceale Bay (4D,E); b: Watchers (8C,D); c: Anxious Bay (6E, 7A,B); d: Point Drummond (12A,C); e: Frenchman (13A,C); f: Fishery Bay (16C). Dotted shading and cross hatching together show the areas fished in 1978 to 1979, and cross hatching the areas fished in 1995 to 1999. Depth contours in meters.

TABLE 3.

Spatial extent of fished areas in eight populations of *H. laevigata* in 1978 to 1979 and 1995 to 1999 with % decline of area fished.

Map Code	Population	Area (1978 to 1979) (km^2)	Area (1995 to 1999) (km^2)	% Decline
4D,E	Sceale Bay	7.3	2.0	68
6E,7A,B	Anxious Bay	87.5	19.1	78
8C,D	Watchers	4.9	1.4	71
12A,C	Pt. Drummond	11.5	4.1	64
13A,C	Frenchman	7.8	1.7	78
15A	Avoid Bay	14.7	0.7	95
16C	Fishery Bay	1.5	0.2	87
25A	Backstairs Pt.	2.0	0.7	65

Keesing et al. 2000), and the exposed habitat of abalone on the bottom at most of the sites ensure high efficiency of capture and the capacity to reduce abundances to very low levels. We also have inconclusive evidence of spatial contraction for other populations: e.g., Highcliff (4A–C), Reef Head (13D–F), and Whidby Is (14E). For many other populations, which occur along a narrow coastal strip, spatial contraction, if it occurred, was at too fine a scale to be detectable by our mapping.

The mapped populations show a striking common pattern in spatial contraction. Fishing areas contracted in every case from deeper, offshore waters toward the coast where they were clustered around headlands, in bays or, in the case of the Watchers, in shallower waters on the lee side of, or between, the islands.

DISCUSSION

Evidence of Overfishing

The assumption is pervasive that the tragedy of the commons (Hardin 1968), in which fisheries collapse under open access policies, will be avoided by the use of extensive input and output restrictions. However, Scott (1993) points out that even with restrictive quotas competitive behavior among fishers can still operate and cause collapses. Although the global catches in each zone give no hint of instability (Fig. 3), the fine-scale analysis tells a different tale. The devil lies in the detail.

The basic problem in this greenlip fishery, maldistribution of effort, in which divers have increasingly competed to overfish the most productive grounds close to port and underfish others was first noted by Prince and Shepherd (1992), but they lacked supporting data on differences in fishing mortality rates, F. However, more recent data on the total mortality coefficient, Z, with distance from port now enable a better quantification of the gradient in F. Estimates of the natural mortality coefficient, M, for greenlip range from 0.22 at Tiparra, 0.38 at West I., and 0.2–0.4 at Waterloo Bay, all inshore sites, to 0.1 at a deep offshore site outside Ward I., which was considered unusually low (reviewed by Shepherd & Breen 1992; Shepherd & Baker 1998). Hence, in the absence of evidence of a gradient in M with increasing distance offshore, we may conclude that F declines from ~0.4–0.6 on near-shore reefs in the Western Zone to ~0.2–0.3 on reefs 40 km from port. The almost universal decline in the catch from near-shore reefs with these high levels of F and the increasing catches at more distant reefs strongly implicate overfishing as the likely cause. But when did overfishing commence?

Increased catches and levels of fishing from 1981 to 1983 were first noted by Lewis et al. (1984), and presumably led to the decline in abundance on inshore reefs in 1984 when divers urgently pressed for a closure of the Western Zone. However, the decline in abundance continued unabated and precipitated the 1989 reduction in quota. The age composition of greenlip in the catch is mainly 6–10 y (O'Loughlin & Shepherd unpublished data), so the consequences of any recruitment overfishing event must be sought some 6 to 8 y later. In retrospect, the most likely explanation for the declines in the late 1980s, evidenced by the peak in the number of populations whose catches had declined by 50% in 1988 to 1989 (Fig. 5), is that recruitment overfishing had occurred from 1981 to 1983. The continuing decline of the same populations that constituted the 80% decline peak in 1995 to 1996 suggests that the quota reduction in 1989 had little or no ameliorative effect.

In the Central Zone with only six divers, the picture is less certain. Reefs were visited by few divers, and in a few populations, catches increased in 1987 to 1988 in anticipation of quotas introduced in 1989 (Keesing & Baker 1998). Hence, changes in abundance must be inferred from time series of catch data varying from 10 to 20 y. Implications of decline inferred from short time series of only 10 y must be tentative until supported by other evidence. Subject to this caveat, the data suggest the widespread decline of Kangaroo I. populations (Map codes 26–32) and increased compensatory fishing pressure at Tiparra (21). Because the Kangaroo I. coast is much more difficult for divers to access than Tiparra, the reverse effect to that seen in the Western Zone occurs; i.e., a movement of fishing away from distant reefs to a near-shore one, where fishing intensity is now high. The reason for the reverse effect is that divers visit Kangaroo I. annually about mid-year when the weather is favorable to take their blacklip quota, and they have historically exploited greenlip stocks during these visits. In recent years, divers have increasingly returned to Tiparra (21) later in the year to complete their greenlip quota, presumably because catches have progressively declined on Kangaroo I. The reasons why declines in the Central Zone seem less serious than in the Western Zone are twofold. First, with many fewer divers in the Central Zone, competition between divers has likely been less, particularly during prequota years, so that overfishing has been less severe. Second, presumed overfishing developed some 6–8 y later in the Central Zone and so has had less time within which to become apparent.

Factors Associated with the Declines

The multiple regression analyses of proportional decline rates are illuminating and suggest major factors affecting the decline of populations. First, we consider the nonsignificant factors. The nonsignificance of IP (initial production) as a factor influencing the rate of decline is consistent with our earlier finding (Fig. 4) that the proportion of populations in serious decline did not change with increasing size of population. This may surprise, as Shepherd and Baker (1998) predicted that small populations would be more vulnerable to overfishing than large ones, because of their higher recruitment variability as found for Backstairs Passage (25A) (Shepherd et al. 2001). One of the assumptions of this analysis is that populations similar distances from port are subject to roughly the same fishing mortality, F. It is possible that smaller populations are visited less often and fished less intensively than larger ones, so that the decline rates of smaller ones would be less than under the assumption of equal exploitation rate. We are currently examining exploitation rates in populations of different sizes to test this possibility.

The other nonsignificant factor in the analysis was depth. However, depth may be relevant at the extremes of shallowness and deep water. At some near-shore shallow sites; e.g., Waterloo Bay, divers have customarily accepted low catch rates while following safety decompression schedules after fishing in deep water, and precipitated a decline (Shepherd & Partington 1995). On the other hand, populations that extend into deep water (>30 m), such as Hotspot (9C,D), Thorny Passage (18C–F), Ward I. (9A,B), and Pearson I. (10C), of which only the last has declined, may find a partial refuge in depth and be more resilient to fishing (Karpov et al. 1998). However, some divers do occasionally fish to 30-m depths and say that deep-water populations have poor recruitment and recover more slowly than shallower populations after fishing. The question remains open until methods are found to survey deeper water.

The significance of D (distance from port) is not surprising, given the gradient in Z with increasing distance from port, and is confirmatory evidence of the maldistribution of effort in the fishery (see above). However, the variable of special interest is the closure index C. This estimates the boundedness of a population by land (or emergent reef) and, by implication, the extent of larval retention within the population. Apart from a very low value of 0.15 for Whidby Isles (14E), where the population collapsed after 6 y fishing, values ranged from 0.30–0.74. We hypothesize that coastal topographic features restrict dispersal of larvae and tend to maintain recruitment or decrease its variability. The hypothesis has substantial support. Wolanski and Hamner (1988) reviewed the effect of coastal topography, such as headlands and islands, on larval transport and noted that topographically generated fronts aggregated eggs and larvae. In empirical studies on this abalone, Shepherd et al. (1992) found that larvae were retained in near-shore eddies and bays, and Shepherd et al. (2001) found that in Avoid Bay (15A) and Backstairs Passage (25C) recruitment was stronger close to shore and weaker off-shore in open habitats, where currents were stronger. Furthermore, the pattern of spatial contraction toward shore around headlands and in bays, found for these populations as well as for six additional populations (Fig. 9), reinforces the evidence in favor of a strong effect of coastal topography on the resilience of greenlip populations. This hypothesis could be tested experimentally by measuring density of larval settlers in collectors in populations with different values of C (see McShane 1995, Rodda et al. 1997). The hypothesis also explains the hitherto puzzling absence of greenlip populations from the many very small islets and sea-mounts that abound within the geographic range of greenlip abalone. Their C values may simply be too low for larval retention.

Spatial contraction of stocks is known for declining fish stocks (e.g., Rose & Kulka 1999), but has not previously been documented for exploited molluscan stocks. The phenomenon may be an example of a much more general principle. Metapopulation models predict that larger metapopulations have higher local abundances than smaller ones, because recruitment rates are higher and extinction rates lower (Hanski & Gyllenberg 1997). The converse; namely, that reductions in density in local populations will reduce the metapopulation range but to a less extent, is expected to follow if recruitment rates declined. In the eight populations in Table 3 the mean percentage decline in catch (90%) is significantly more than the mean percentage spatial decline (76%) ($t = 2.4$; $P < 0.05$), suggesting that, on average, density declined more than the spatial area occupied, as predicted by theory. However, other models not based on metapopulation theory and with other assumptions may predict the same result (Rosenweig 1991, Holt et al. 1997, & see Lawton 2000).

Alternative Explanations for the Declines

The major underlying assumption of the above analysis is that the total catch reflects abundance. Jamieson (1993) noted the tendency in capture fisheries for divers to move to new reefs only when exploited ones are exhausted, and McShane (1998) observed the preference of divers to fish reefs closer to home. Keesing and Baker (1998) relied on these arguments in their earlier analysis of this dataset to interpret trends in abundance of local stocks. Shepherd et al. (2001) reviewed diver behavior in the context of abalone fisheries and concluded that the diversity of diver exploratory behaviors and the single dominant motivation of all divers; namely, maximization of catch rates, ensured complete coverage of every reef and efficient removal of abalone aggregations. Hence, the total catch should crudely reflect stock abundance in fully or near fully exploited stocks, although the relation may not be linear where spatial contraction of the stock occurs. Empirical evidence from Avoid Bay (15A) and Backstairs Passage (25A) show a curvilinear relation between survey densities and catch (Shepherd et al. 2001). However, in stable populations, there is no such correlation between catch and density (e.g. Tiparra Reef (21): $r = -0.10$, $n = 18$; Thorny Passage (18C–F): $r = -0.39$, $n = 11$; Ward Island (9A,B): $r = -0.07$, $n = 18$; Hotspot (9C,D): $r = 0.28$, $n = 17$—see Shepherd et al. 1999). This result is not surprising, because surveys are not coordinated with fishing that may occur before or after an annual survey (Shepherd & Baker 1998). Thus, there is an asymmetric relation between catch and abundance in which the catch reflects abundance but only in declining populations. Nevertheless, it cannot be concluded that a declining catch is, of itself, proof of declining abundance. This is the well-known fallacy of *affirming the consequent*. In formal terms, declining catch is a necessary, but not a sufficient, indicator of declining abundance. The alternative, testable hypothesis—that a population is stable but the catch declines for reasons unrelated to abundance—must now be considered.

Two versions of this hypothesis have been proposed by industry and managers to account for the observed trends in the catch. First, it is suggested that changes in management since 1979 could have caused the patterns seen. The notable changes were: saleability of licences, size limit increase, and short-term closure (Western Zone only), quota imposition, and quota reduction (Western Zone only), (see above). The argument ignores history and fails to distinguish cause and consequence. The declines in catch from 1984 to 1988 in the Western Zone were real and pervasive and led to strong pressure from divers to introduce quotas and increase size limits and also led to increased government concern in 1988, which was followed by the quota reductions in 1989. Size limit increases could not have caused catch declines, because they were largely neutral in respect to yield (Shepherd & Baker 1998). Furthermore, if management measures up until 1989 caused the catch declines, they would not have continued unabated into the 1990s (see Fig. 5), but would have stabilized or oscillated within stable limit cycles as observed in the Far West (Keesing et al. 2000).

The second version of the hypothesis is that the pattern of fishing has gradually changed over time, not as a response to declining abundance, but for reasons as diverse as: occasional presence of sharks, higher catch rates at offshore islands and its variant, the use of Global Positioning Systems, and preference for clear rather than dirty water.

We term both versions the underfishing hypothesis, because a necessary implication of each is that *underfishing* of populations with declining catches is occurring. If a population is stable with a surplus available for capture, then the capture of less than the surplus will lead to an increase in population size. Moreover, the greater the decline in catch and the longer it has been declining, the greater would be the increase in numbers and biomass, until eventually, the population would approach virgin densities, and Z would approach the natural mortality rate M. This hypothesis can be variously tested by research diver surveys, evidence of spatial contraction, and estimation of Z, from catch-curve analysis. The combined cumulative evidence in this paper of low densities from survey data, spatial contraction of populations and/or high Z values for twelve problematic reefs with declining catches (see Table 1) together point to widespread overfishing, inconsistent with the underfishing hypothesis.

A satisfactory explanatory hypothesis must be able to withstand tests of internal coherence, external consistency, and predictive accuracy (Giere 1999). Since 1995 when major declines were first detected, overfishing of specific reefs has been conceded as data have accumulated, but only as exceptions to an otherwise stable fishery. A hypothesis is incoherent if every contradiction constitutes an *ad hoc* exception to it.

Is the underfishing hypothesis externally consistent with other statements? Historically, surveys of divers, increasingly accepted as a valuable source of information at a local scale (see Neis et al. 1999), have provided valuable evidence on divers' perceptions of the status of reefs. Surveys of divers of the Western Zone during 1995 to 1999 (see Shepherd 1996 for a progress report) showed that divers' perceptions were cumulatively in unanimous agreement with inferences of decline drawn from catch data for every reef from Fishery Bay (16C) north to Sceale Bay (4D.E), except for Sceale Bay itself where opinions were divided. (Each diver was familiar with only a small subset of the reefs.) Divers judged the productivity of reefs by the frequency of their visits, a fact that has long been known. In an early perceptive submission to government the divers' association wrote: "When abalone start to get hard to find in an area or a diver knows of somewhere he may do better, he moves on, and in this way rotates the areas and allows them to rest. The divers find that they are rotating their areas faster each year. . . . Even though they are able to maintain their catch rate the areas are not supporting the previous year's effort." (Letter from Abalone Divers Association to Minister for Agriculture and Fisheries dated 18 October 1976.) Thus, from the relatively early days of fishing, divers recognized that decision thresholds for ceasing to fish were a response to low abundance (see Shepherd et al. 2001 for further discussion). Last, if low abundance is not the basis of the decision to cease fishing a reef, no other credible cause has been proposed that coordinates the behavior of 29 idiosyncratic divers to underfish or cease fishing reefs in entire geographic regions for a decade or more. Thus, the underfishing hypothesis is counterbalanced or refuted by many other statements of divers and lacks credibility.

Last, does the underfishing hypothesis have predictive accuracy? As shown above, the hypothesis has been tested at twelve reefs and failed. Although testing the hypothesis at other reefs is an ongoing program, the underfishing hypothesis, so far, has failed every test of coherence, consistency, and predictive accuracy. It is, moreover, value laden, because it is proposed on behalf of those with a strong economic interest to maintain quota levels.

A third hypothesis is that long-term environmental changes have affected population persistence in this abalone. Coastal waters of South Australia are influenced by seasonal upwellings from January–March each year, but this occurs well after the spawning season in October–November (Shepherd & Laws 1974) so is an unlikely agent directly affecting recruitment. Even if it affected adults, the upwellings extend up to 60 km from the coast (Griffin et al. 1997) so the selective decline of populations in two zones and very few in the far west are difficult to explain. Moreover, the evidence of long-term temperature changes in the region is slight. Hence, this hypothesis seems untenable.

In contrast, the overfishing hypothesis provides a common explanatory mechanism for the decline of catch on many disparate reefs and also suggests new areas for further research. One is that coastal topography influences population persistence via mechanisms of larval retention. This will be a useful criterion in the search for, and protection of, larval source habitats and in determining sustainable exploitation rates of reefs.

Management in Review

The historical trend in management from government to fisher control, as exemplified in this fishery, is, in the view of Scott (1993), part of a broad endogenous movement, not unique to fisheries, whose endpoint, at least for territorial stocks, is local self-management by fisher groups and cooperatives. Within fisheries, a general theory of government–community comanagement is emerging from the reviews of Jentoft (1989), Scott (1993), Pinkerton (1994), Wilson et al. (1994), Jentoft and McCay (1995), Sen and Raakjaer Nielsen (1996) and Hutton and Pitcher (1998). Almost all of these reviewers argue that it is the role of government to set quotas and of the management committee to "decide the how, when, and where of fishing" (Kesteven 1995). The present self-management system for the abalone fishery displays many of the positive features, such as flexibility, responsiveness, equity, and homogeneity of interest reducing internal conflict, considered by these authors and also lacks many negative features, such as deficits of information and excessive numbers of fishers. Fixing quotas is properly reserved to government. However, are there deficiencies in the present system as it has moved toward self-government?

The influence of government in the AMC seems to have declined relative to the power of the major interest groups since 1995, especially in matters relating to the conservation of stocks. Management has not applied the precautionary approach (PA) as mandated in the management plan (Zacharin 1997) nor addressed the maldistribution of effort by closures and other means. It might be argued that management has been unwilling to make hard decisions or that too much power has passed to an industry increasingly dominated by economic interests. It might be claimed, in defence of inaction, that the inherent uncertainty of assessment, the absence of satisfactory reference points (Shepherd et al. 2001), or the insufficiency of the evidence have not required action even under the PA. These issues are complex and controversial and will be considered elsewhere. Our purpose, here, is to highlight the basic problem and suggest a way forward. The problem of possible conflict of interest in the AMC could be met structurally by more effectively divorcing day-to-day management from the fixing of quotas. Referral of the quota-setting function to an independent quota committee of experts, as operates in New South Wales, would facilitate independent and objective appraisal of the evidence. The problem of uncertainty of assessment must be ad-

dressed by accumulating more and stronger evidence, developing an agreed set of indicators for the fishery (Richards & Maguire 1998, Shepherd et al. 2001) and applying it to stocks at risk. In the meantime, application of the PA, incorporating understanding achieved from monitoring known, collapsed stocks, could minimize the risk of further overfishing.

Precautionary Approach (PA)

Although this study does not establish conclusively the serial decline of the greenlip fishery as a whole, is the evidence sufficient to invoke the PA? The PA (reviewed by Foster et al. 2000) reverses the onus of proof and states (Principle 15 of Rio Declaration as applied to fisheries) that where there are threats of serious damage, lack of full scientific certainty shall not be used as a reason for postponing measures to prevent overfishing. For data-poor fisheries (such as this one in which nothing is known of >90% of the stocks), respective FAO and U.S. national guidelines for application of the PA (Garcia 1994, Garcia 1996, Restrepo & Powers 1999) advocate conservative measures, including:

1. setting catch limits based on qualitative judgements about stock status and stock rebuilding strategies for stocks *believed to be* overfished, within time frames of 10 y (our italics); and
2. establishing provisional reference points by analogy to similar and better known stocks.

They also recommend that in situations of doubt, scientists analyzing management options should systematically analyze and highlight the most pessimistic scenarios. Dayton (1998) argues that reversal of the onus of proof is the only mechanism by which action can be achieved, given the penchant for regulators to procrastinate by "creating imaginative, alternative explanations" or simply by asserting "that the data are incomplete" (Dayton 1998). Given the coherent, cumulative evidence for a pervasive overfishing scenario there seems a proper case for application of the PA to this fishery. In the terms of Orensanz et al. (1998), overfishing "has to be considered as the default working scenario, even before being tested as a scientific hypothesis."

Signs of serial decline of this greenlip fishery were first noted in 1995 (Shepherd 1996, Keesing and Baker 1998), and a mechanism for decline proposed by Shepherd and Baker (1998). The apparent continuing decline toward commercial extinction of many stocks finds a parallel in the decline of about a third of the Canadian salmon stocks (Riddell 1993). As emphasised by Pinkerton (1994), dependence of a fishery on fewer stocks is risky, because effort is increasingly focused on the remaining stocks and will increase the risk of their decline. On the other hand, conserving more stocks is a buffer against future recruitment failure, which could simultaneously affect many stocks.

For the conservation of sedentary invertebrate stocks, Jamieson (1993) advocated:

1. very high minimum size limits;
2. unexploited refuges;
3. maintenance of high densities in many areas; and
4. specific management of metapopulation units.

In this fishery, high size limits have been advocated by Shepherd and Baker (1998), creation of refuges and management of metapopulations by Keesing and Baker (1998), who also suggested multiple size limits and temporal closures to control yields. The establishment of small fishery reserves (Baker et al. 1996, Nowlis & Roberts 1999) in areas where relict subpopulations persist, supplemented by adult transplants, would allow aggregations of adults to accumulate and provide a source of larval recruits. A multiplicity of measures applied at a fine scale over large areas will be necessary to restore the former productivity of the fishery.

The desired outcomes of comanagement are that sustainability, efficiency, and equity in managing the resource will be improved (Sen & Raakjaer Nielsen 1996). However, if the first of these fails, the rest no longer matter. Although management of this fishery may have enjoyed some success on the last two outcomes, the first is now at risk.

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A CHRONICLE OF COLLAPSE IN TWO ABALONE STOCKS WITH PROPOSALS FOR PRECAUTIONARY MANAGEMENT

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ABSTRACT Two populations of greenlip abalone, *Haliotis laevigata*, in Backstairs Passage and Avoid Bay, collapsed during 30 years of fishing. The former population was monitored from 1980 to 1999, and the latter from 1987 to 1999. Both populations showed strong spatial contraction as their respective subpopulations in open habitat failed first compared with those in inlets, bays and around islands, where recruitment was higher. Stock-recruitment curves are gently sloping or indeterminate, indicating weak density-dependence in the one case and high variability in the other case. From data gathered during the decline of these populations we review various fishery indicators which may be useful for monitoring greenlip abalone populations and for predicting collapse. Total catch was correlated with relative spawner abundance from survey data, and thus appears to be a useful indicator of abundance in this fishery. Other useful indicators are densities and size compositions, from which annual recruitment strength and total mortality can be derived, and survey evidence of spatial contraction of fished areas. Egg-per-recruit analyses are also useful to estimate the extent of departure from predetermined safe levels of egg production. Given the fuzzy knowledge of the status of populations, several indicators are best used in concert to determine management strategies. We propose a "troublespot thermostat" approach in which a suite of indicators, sequentially triggered off, call for increasingly severe management responses. In this way declines can be detected and arrested, and timely steps taken to avoid collapse.

KEY WORDS: population ecology, stock-recruitment, spatial contraction, fishery collapse, fishery indicator, biological reference point, abalone fishery, *Haliotis laevigata* egg-per-recruit

INTRODUCTION

While a legislative framework to achieve the ecological sustainability of fisheries has been in place for some years in Australia, management has either been weak or has lagged seriously (Nicholls & Young 2000). The principle risks degenerating to empty rhetoric unless rigorous criteria are developed to detect and arrest fishery declines at an early stage. In particular robust biological reference points to indicate declines have been developed for very few fisheries.

The fishery for greenlip abalone (*Haliotis laevigata* Donovan) started in about 1966 in southern Australia and was controlled in the different States initially by input measures and then by quotas progressively introduced in the 1980s (Prince & Shepherd 1992). Greenlip populations have been slowly declining in the last two decades, first in Victoria and progressively in Tasmania and South Australia (Officer 1999, Shepherd & Rodda 2001). Management response to the declines has been tardy for many reasons. The absence of clear fishery indicators has promoted uncertainty, continued high catch rates have engendered skepticism about overfishing concerns, and the propagation of the notion that a stock-recruitment relation in abalone "is largely uncorroborated dogma" (McShane 1995) has favored complacency.

Catch data from the South Australian abalone fishery were collected from 1968 at a coarse scale of 37 × 37 km, and from 1979 at a finer (metapopulation) scale (Sluczanowski 1986, Keesing & Baker 1998, Shepherd & Rodda 2001). Data collection at the fine scale enabled researchers from about 1995 to follow long-term trends in individual populations. Annual monitoring of commercially exploited populations started in 1980 (Shepherd & Baker 1998) at a few sites and was gradually extended to many others. Two of these populations, monitored during part of their fishing history, collapsed during the 1990s and this article describes their decline. We document the spatial contraction of the stocks, provide length compositional data and estimates of the total mortality coefficient, Z, obtained during the decline phase, and the decline in density of spawners and recruits. We present spawner-recruit curves and evaluate various fishery indicators of possible use in abalone fisheries. In the absence of unambiguous indicators of overfishing, we suggest a protocol, which we term a trouble spot thermostat approach, by which population declines can be detected early, data assembled, and declines arrested by appropriate management measures before final stock collapse when recovery becomes problematic. In this article we use the terms population and stock to mean an inferred metapopulation (Shepherd & Brown 1993, Shepherd & Rodda 2001), following the detailed genetic studies of Brown and Murray (1992) on greenlip abalone.

MATERIALS AND METHODS

Site Descriptions

Backstairs Passage

Backstairs Passage lies at the entrance to Gulf St Vincent (see map in Shepherd & Rodda 2001). Greenlip abalone populations occur discontinuously on boulder or reef bottom over an area of ~2 km^2 from several km east of Blowhole Creek to a little north of Cape Jervis on the Fleurieu Peninsula, South Australia (Fig. 1a). Rocky substratum is Cambrian greywackes and schists, tilted at a sharp angle providing microhabitat for juvenile abalone. Further offshore, boulders lie scattered on sandy or rocky bottom (Keesing et al. 2000). Abalone habitat is narrow at the southern end but broadens toward Fishery Bay and Cape Jervis where rocky bottom extends continuously or interspersed by seagrass patches to nearly 1 km from shore to a depth of 12–13 m. Highest densities occur near the entrances to the numerous inlets along the coast, in Fishery Bay and north of Lands End.

The Passage is swept by tidal currents, which reverse twice daily due to diurnal and semi-diurnal components (Bye 1976,

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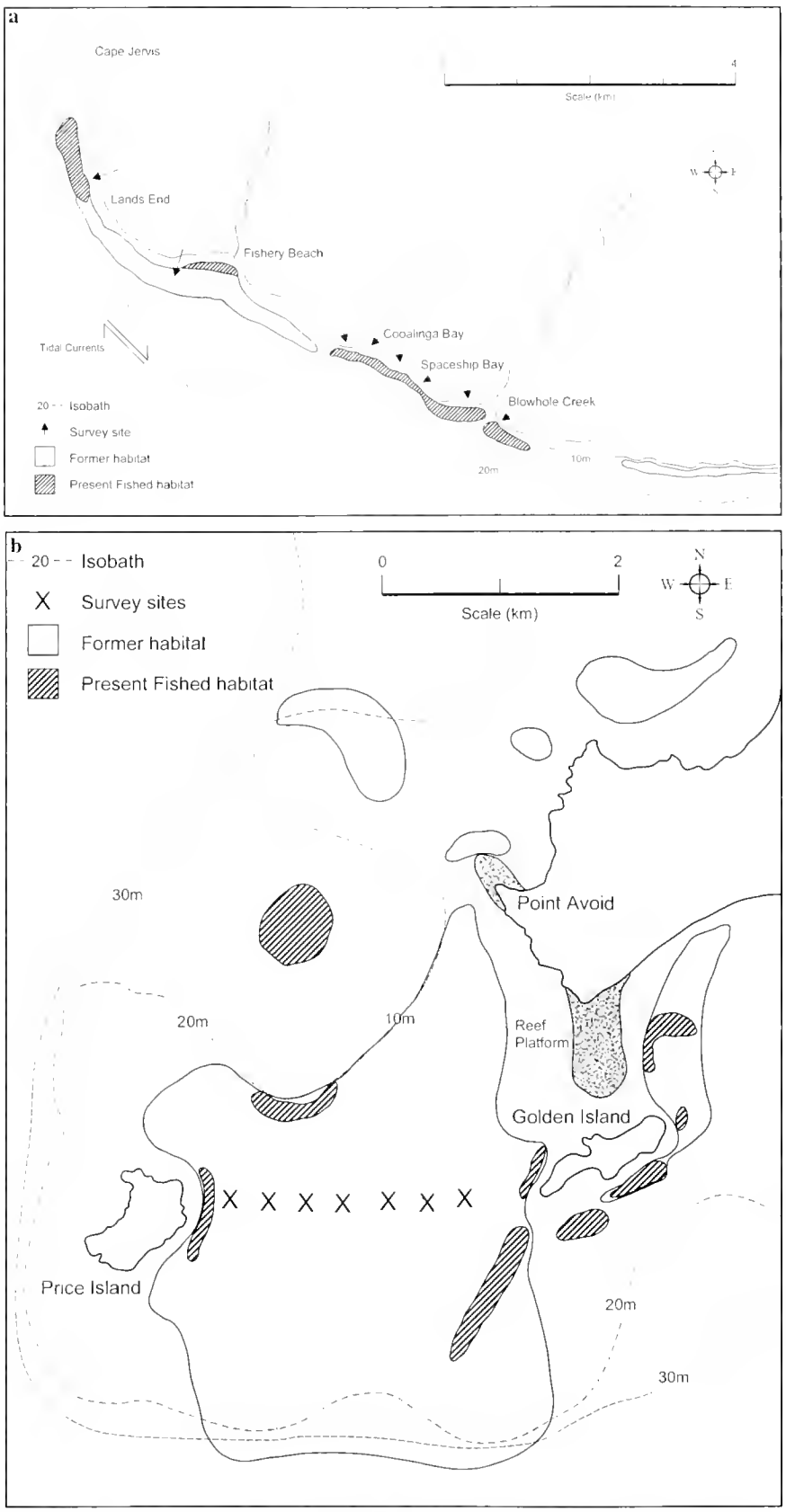

Figure 1. (a) map of greenlip abalone habitat in Backstairs Passage showing fished areas in 1978 (light shading), and in 1999 (dark shading). Survey sites are marked with arrows. (b) map of greenlip abalone habitat off Point Avoid showing fished areas in 1978 (light shading) and in 1999 (dark shading). Very dark areas are intertidal reef platforms. Survey sites 1–7 are shown by crosses, and numbered 1 to 7 from left to right.

Bowers & Lennon 1990). During neap tides, which occur every 14 days, when tidal movement is minimal, water velocities are 35 cm sec^{-1}, increasing to 1.4 m sec^{-1} seven days later during the spring tide (Carvalho & Bye 1996). The maximum tidal excursion is > 6 km during a neap tide and > 20 km during a spring tide. Close inshore on the coast of Fleurieu Peninsula currents are slower, and in the inlets from Blowhole Creek to Fishery Bay and north of Lands End (Fig.1a) there are reverse currents or eddies. There is a mean annual residual flow out through Backstairs passage of ~10 cm sec^{-1}, suggesting a net transport of larvae southeasterly, unless the net flow is reversed by southerly winds.

Fixed sampling sites were established in 1980 at the southern, central and northern sections of the population, namely at Blowhole Creek in the south, Spaceship and Cooalinga Bays in the center, and in Fishery Bay and Lands End in the north. Rocky bottom was continuous at the sampling sites and divers either followed a constant direction or in the case of the southern sites followed the sand-rock interface.

Avoid Bay

Abalone habitat of gneissic or calcrete substratum extends over an area of about 14.7 km^2 from the 30 m isobath south of Pt Avoid between Price and Golden Islands mainly at depths of 15–25 m northwards toward Pt Avoid, with smaller local populations northwest to northeast of Pt Avoid and northeast of Golden I. (Fig. 1b) (Keesing et al. 2000).

From drogue studies at similar coastal sites we estimated that maximum tidal currents which reverse twice daily with a north/south flow are ~20 cm sec^{-1}, and that the maximal tidal excursion is ~4 km. These estimates may be extreme due to the dampening effect of swell on water currents (McShane et al. 1988). Seven survey sites were established at 250-m intervals between Price and Golden Islands at 17–21 m depth (Fig. 1b).

Monitoring Procedures

Monitoring of greenlip populations started in Backstairs Passage in 1980 and in Avoid Bay in 1987. For each population we chose representative sites according to the known distribution of the populations and subject to time and depth constraints. The sampling was systematic to minimize spatial variability, and sampling sites were fixed by reference to land marks in Backstairs Passage and by radar or Geographic Positioning System (GPS) in Avoid Bay. We used the free-swimming, timed swim technique (Kenchington 1978, Shepherd 1985) to measure the relative abundance and size distribution of greenlip *in situ*. Divers measured all abalone encountered with an underwater measuring gauge, in which data points of shell length (SL) are imprinted onto plastic (Shepherd 1985), within a swathe of 1m for 10 min. Surveys were done in the summer and fall from November to May.

The number of replicate 10-min swims per site varied according to the conditions and number of divers available. In Backstairs Passage the mean replication was 5.3 (range 3–9) in the southern section, 8.1 (range 3–16) in the central section and 6.7 (range 2–12) in the northern section. In Avoid Bay there were four replicate 10-minute swims per site. For Fishery Bay we lacked data for the years 1982, 1985 and 1988. For the purpose of calculating mean values for Fig. 6 we estimated recruitment (i.e. the density, D_3, of the 3+ age class) in those years from the density, D_4, of the 4+ age-class in the following year at the site from the equation $D_3 = 0.44\ D_4^{2.77}$ (range of D_4 values 0–1.9/100 m^2) derived empirically over 10 y from all sites combined in the central section where we had the most data. For the same years we interpolated the density of the spawning stock as the mean of the values for the preceding and succeeding year. The above equation has no implications about survival of the D_3 age-class due to the effect of changing sighting probabilities of recruits over time (Shepherd 1990).

Data Sources and Analysis

Catch data, presented in total (in-shell) weights, (TW) are derived from the catch and effort monitoring system in place since 1968. Backstairs Passage is in the Central Zone where there are six licensed divers and Avoid Bay is in the Western Zone with 23 divers. Some poaching in Backstairs Passage was reported occasionally during the 1980s but not since. Backstairs Passage has been predominantly fished by only one or two divers since the mid 1980s. About half the catch from Avoid Bay was graded by size during processing. The three grades, in terms of number of meats per unit weight, are approximately (after metric conversion): 1: <4/kg; 2: 4–6/kg; 3: >6/kg. The data are given in full in Keesing et al. (2000).

Juvenile *H. laevigata* are cryptic to an age of two and a half years then gradually emerge with size (Shepherd 1990, Shepherd & Partington 1995), so we used the 3+ age-class as an index of

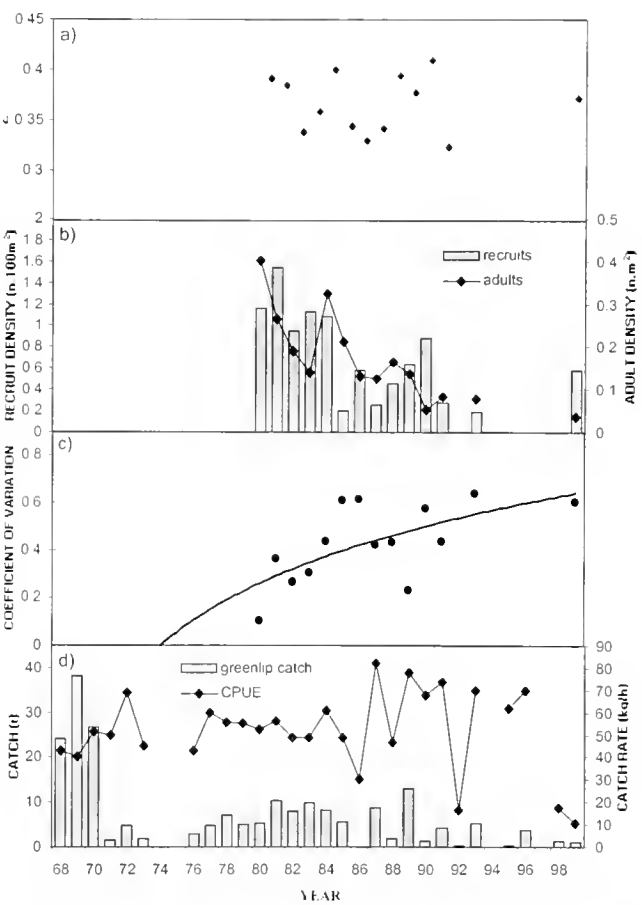

Figure 2. Catch/effort and survey history of the Backstairs Passage greenlip fishery showing: (a) change in total mortality coefficient, Z, over time; (b) mean adult and recruit (3+ age-class) densities for survey sites from 1979–1998; (c) change in coefficient of variation of mean recruitment at survey sites over time, with a trend line fitted to the data; (d) total catch (t TW) and catch rate in kg/hr from 1968–1999.

recruitment strength, and estimated its size range at the date of sampling from the known growth rate. The growth rate of *H. laevigata* is linear during the first 4 y of life in Backstairs Passage (21.3 mm/y) and at Avoid Bay (22.2 mm/yr) (unpublished data, Shepherd & Triantafillos 1997) and we assumed a uniform birthdate on 1 December each year. We used knife-edge separation from the 2+ and 4+ year classes to estimate their abundance in length-frequency samples.

In order to estimate density from the timed swim data we used the equation relating area covered, A, to swimming time, T, number of abalone measured, N, measuring time, m, and diver power, r, given by Shepherd (1985):

$$A = Tr - mrN$$

Swell and algal cover were low at all our sampling sites so we modified the original equation given by Shepherd (1985) to exclude effects of swell and algal cover on diver power.

As the individual power and measuring time of some of the divers used in the surveys were not calibrated (although all were experienced in abalone survey work) we used mean values of r = 20 m^2 min^{-1}, and m = 4 sec (see Shepherd 1985) to calculate the area covered per 10 minute swim. Mean recruitment density (D_3) and adult densities were estimated for each site and the mean for all sites gave the mean annual population values.

Recruitment variability was measured as the coefficient of variation (the standard deviation of the mean annual recruitment values for all sites divided by the mean) and expressed as percentage values (Pimm 1991).

Estimates of the original reef area fished for abalone were obtained from information supplied by commercial divers in 1977 to 1979 to one of us (SAS). We drew maps during interviews with three divers for Backstairs Passage and four for Avoid Bay, and the resultant map was the common area described by the divers for Avoid Bay, but a composite map for Backstairs Passage, which included our own survey data.

Total mortality rates, Z, were obtained from catch-curve analysis. Commercial samples of shells (>450 shells per site), taken throughout the fishing area, were aged (O'Loughlin & Shepherd unpublished data) with the shell-aging technique of Shepherd and Triantafillos (1997) and an age-length key prepared for each site. Length-frequency distributions were then converted to age-frequency distributions and Z estimated from the Chapman and Robson (CR) (1960) estimator, preferred by Dunn et al. (1999) to regression methods. The equation used is:

$$CR = \mathrm{Ln}\,(1 + a - 1/n)/a$$

where a is the mean age (above the recruitment age) and n is the sample size. The analysis assumes a stable age-length structure. This seems reasonable for this abalone, given the absence of evidence for inter annual variability in growth rates (Shepherd & Hearn 1983).

To estimate the density of the spawning stock in Backstairs Passage for the missing year, 1996, we used the empirically derived curvilinear relation between total catch and adult density given in Results. Similarly we estimated the density of the spawning stock in Avoid Bay for 1988 from the respective equation for Avoid Bay (see Results).

In this paper the spawning (or adult) stock is the number of

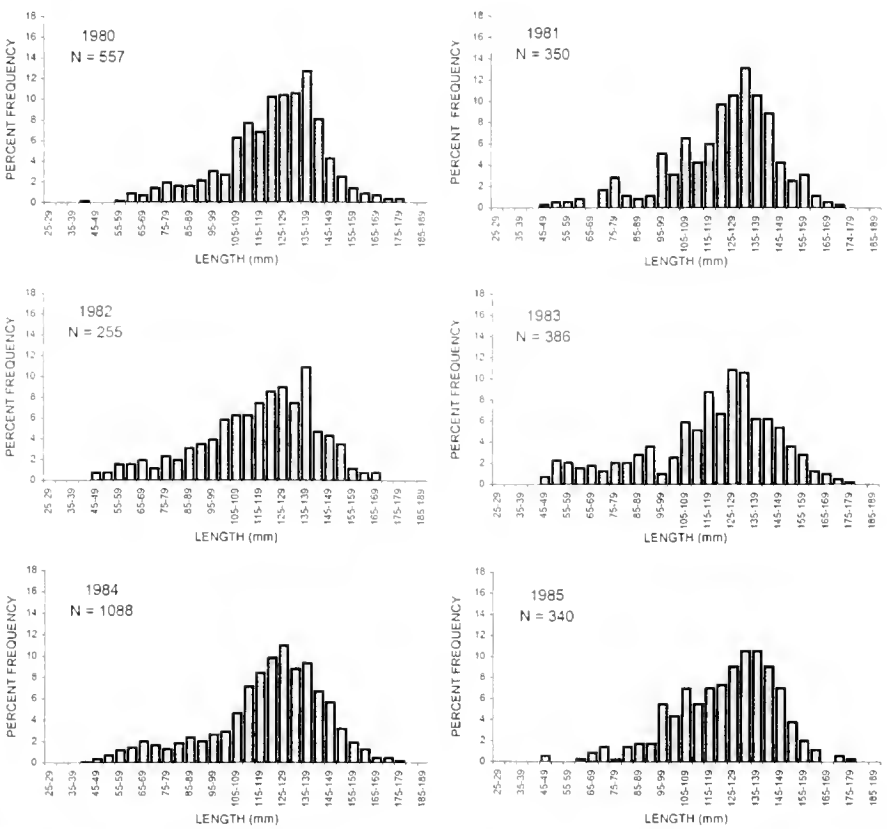

Figure 3. Backstairs Passage. Length frequency data from annual research surveys for all sites combined. The 1993 survey was interrupted by bad weather.

individuals ≥4 years of age i.e. ≥85 mm for Backstairs Passage and ≥90 mm for Avoid Bay.

RESULTS

Backstairs Passage

The catch history of the Passage shows high initial catches in the first three years, typical of a virgin stock, followed by a sharp decline in the catch, and then irregular pulse fishing until the population collapsed after about 1990 (Fig. 2). The catch rates remained high, with occasional falls until 1998 when it fell sharply to a low rate. The fall was due, according to the main diver then working there, to his replacement by an inexperienced diver.

Survey data from 1980 to 1999 provide more detail about the mechanism of decline. Length-frequency data pooled for all sites within years (Fig. 3) showed little change during the period of monitoring, and estimates of Z derived from them also varied little around a mean of 0.37 (s.e. 0.01, range 0.32–0.41), with no downward trend as density declined (Fig. 2a). Mean adult density declined more or less continuously until 1993, and then leveled out, but by 1999 had fallen even lower (Fig. 2). Recruit density varied in an apparently cyclic manner but showed a long-term downward trend, and recruitment variability increased, as shown by the significant ($r = 0.65$; $P < 0.05$), long-term, upward trend in the coefficient of variation (CV).

Local populations at survey sites did not decline uniformly (Fig. 4). The Fishery Bay subpopulation failed first following a steep decline in 1985 to 1987, and by 1993 greenlip abalone were virtually extinct there, except on inshore reefs where small numbers still persisted even in 1999. The spatial contraction of the subpopulation in Fishery Bay and that east of Blowhole Creek (Fig. 1a) was ~115 ha. The remaining populations declined more or less linearly, at a mean annual rate of 3.8% for the southern sites and 5.8% for Lands End. The difference in decline rates was significant ($t = 3.4$; $P < 0.05$). Spatial contraction also occurred in these subpopulations as they retreated toward the coast, but we have not attempted to estimate the areas involved.

The total catch, C in tonnes TW, smoothed with a 3-y running average, shows a curvilinear relation with adult density, D in numbers/m^2 (Fig. 5). The equation of best fit is:

$$C = 10.03 + 2.46 \ln D \qquad (R^2 = 0.36;\ P < 0.05)$$

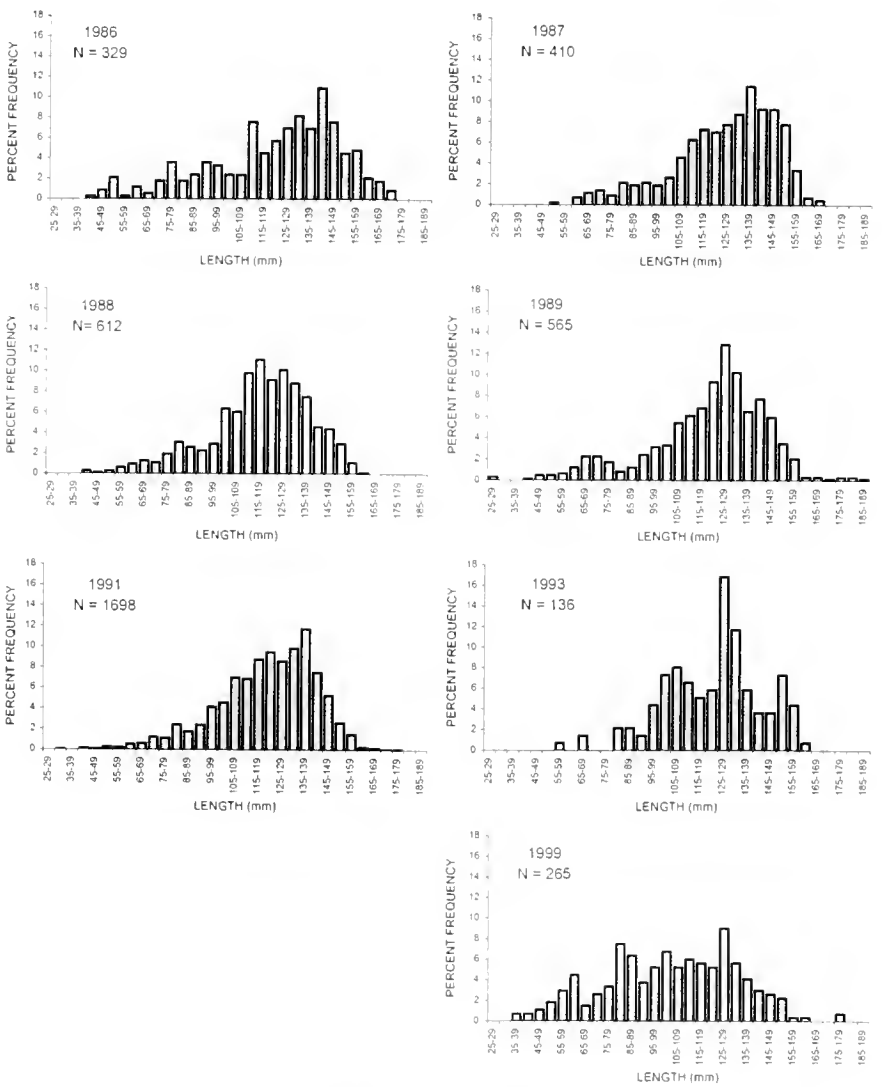

Figure 3. Continued.

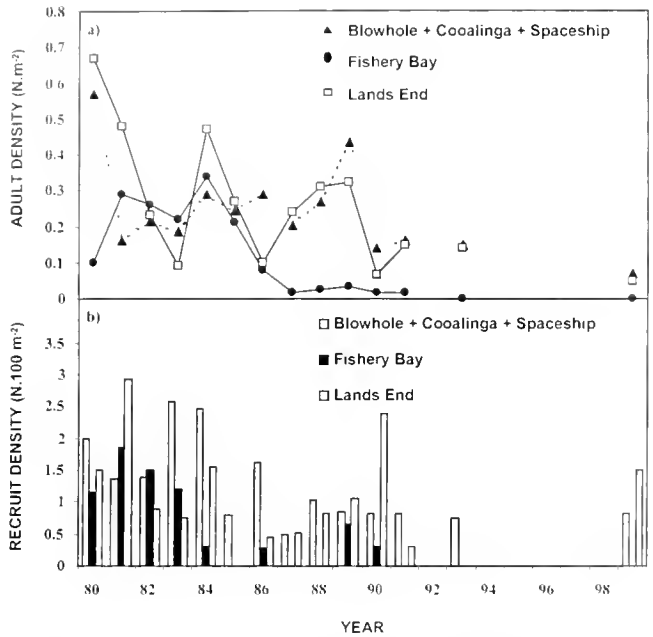

Figure 4. Backstairs Passage. Survey densities of (a) adults in numbers/m^2, and (b) recruits in numbers/100m^2, from 1980–1999 in three sections of the population: Blowhole Creek to Spaceship Bay, Fishery Bay, and Lands End.

Assuming that the Backstairs Passage population is isolated from other populations i.e. without external larval sources, and that larvae from component subpopulations enter a common larval pool (see Discussion), we can examine recruitment in relation to spawner abundance. Stock-recruitment (SR) curves for (a) data from all sites combined and (b) for the Fishery Bay population alone (Fig. 6) show lower curves for Fishery Bay compared with that for all sites, consistent with the more frequent recruitment failures at Fishery Bay. We used the same annual densities of spawning stock for both curves on the assumption that the Fishery Bay subpopulation was linked by larval dispersal to other subpopulations in Backstairs Passage. The equations of best fit are:

$$R_{all} = 1.07 + 0.28 \ln D \qquad (R^2 = 0.19)$$

for all sites combined and:

$$R_f = 0.77 + 0.28 \text{ Ln } D \qquad (R^2 = 0.16)$$

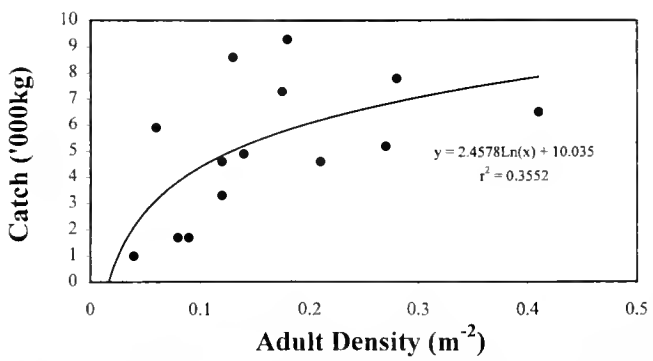

Figure 5. Backstairs Passage. Plot of total greenlip catch in tonnes TW vs adult density in numbers/m^2 with curve of best fit.

for the Fishery Bay subpopulation. In these equations R_{all} and R_f are the mean density of the 3+ age-class for all sites, and for Fishery Bay, respectively, in numbers/m^2 and D is the mean adult density for all sites in numbers/m^2. The data points for 1977 to 1999, for which density was estimated from the catch, are shown in Figure 6 as hollow symbols. These estimated points were excluded from the regression analyses, but are included to show that strong recruitment from the earlier years of the fishery may have produced more elevated or even dome-shaped curves. Note that no strong recruitment occurred at mean densities of <0.15 /m^2.

Avoid Bay

We do not have data at a reef scale for the catch from 1968–1970 when virgin stocks were removed, but the subsequent mean annual catch in Avoid Bay for years 1971 to 1978 was 49.1 t (Keesing et al. 2000). From 1979 to 1983 the annual catch was on average 14% higher than the previous eight years and then declined sharply from 1984 until 1998 when the catch was ~1 t (Fig. 7), giving a mean annual rate of decline of 5.8% since 1979. Catch rates in Avoid Bay remained more or less constant, save for a slight decline in 1996.

Surveys of the Bay started in 1987 when the catch was already well in decline. Length-frequency data (Fig. 8) show little change over the period, and estimates of Z similarly are within a narrow range of 0.50 to 0.67 with a slight but non-significant downward trend (see Fig. 9a). Adult densities, initially two to three times higher at the inshore Sites (1–4) than at the offshore sites (5–7), declined more or less continuously at both, so that from 1996 offshore densities were only ~0.05.m^{-2} and too low to be worth fishing. Density of recruits showed only a weak downward trend over time at Sites 1–4, while at Sites 5–7 recruitment was low, variable, and not detectable after 1995. The coefficient of variation of recruitment (Fig. 7b) increased until 1993 and thereafter fluctuated widely. Although survey data are lacking for the most productive period of the fishery (1971–1983) it is clear that adult populations failed at the offshore sites earlier than they did at the inshore ones. Spatial contraction of the stock was very marked (Fig. 1b), with a total loss of fishable habitat of ~14 km^2. The only places now fished are those close to Price and Golden Islands and near Pt Avoid.

Plots of changes in proportional abundance of three size grades

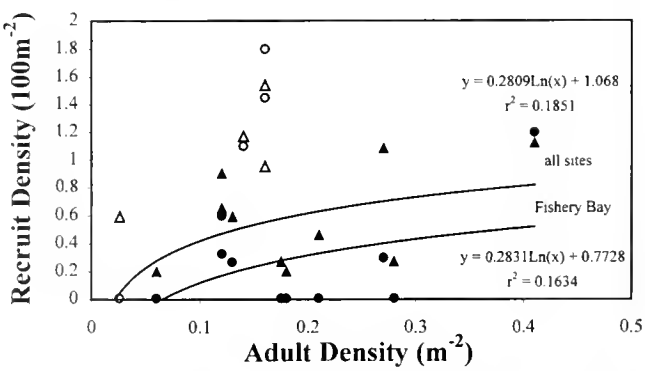

Figure 6. Backstairs Passage. Stock-recruitment curves for all sites combined and for Fishery Bay alone with curves of best fit. The mean annual density of recruits (3+ age-class) is plotted against the mean density of adults 3 y earlier. Recruit densities are: solid triangles (all sites), solid circles (Fishery Bay) and hollow symbols for the years 1977–9.

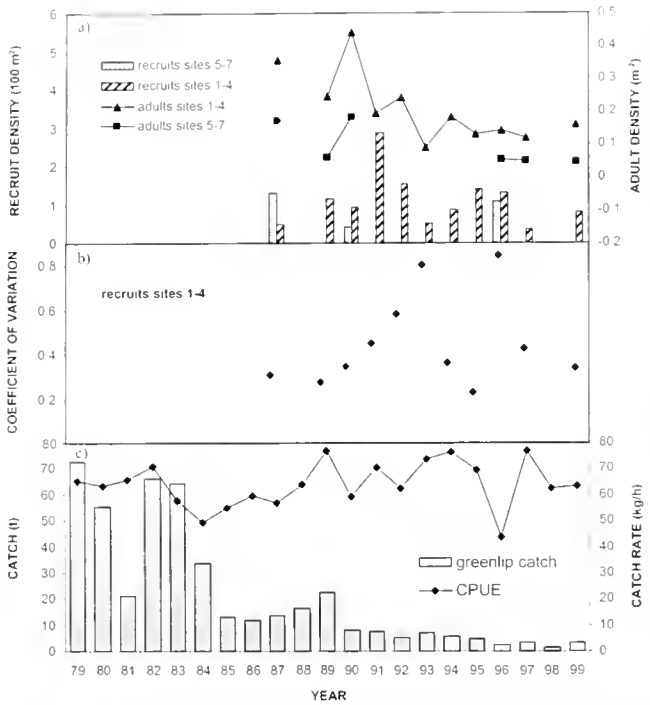

Figure 7. Catch history of greenlip abalone in Avoid Bay showing: (a) mean adult (numbers/m^2) and recruit (numbers/100 m^2) densities for 1979–1999; (b) change in coefficient of variation of mean recruitment for sites 1–4; (c) total catch (t TW) and catch rate in kg/hr for 1979–1999.

over time (Fig. 9b) show that the largest grade (Grade 1) increased significantly ($P < 0.001$), and the smallest (Grade 3) decreased significantly ($P < 0.05$) over the 21 y, but this may have been contributed to by the size limit increase in 1984. If we exclude data prior to 1984 then the same trends in grades are apparent but they are not significant ($P > 0.05$).

The total catch, C, smoothed with a 3-y running mean, shows a curvilinear relation with adult density, D (Fig. 10). The equation of best fit is:

$$C = 20.57 + 6.76 \ln D \qquad (R^2 = 0.63;\ P < 0.05)$$

Plots of mean recruitment density for nearshore and offshore sites vs mean adult density (Fig. 11) show very variable recruitment in relation to adult density at nearshore sites and generally poor recruitment for the few offshore data points. The shape of any SR relationship appears indeterminate so we simply show the line of best fit for each group of sites forced through the origin. The curves imply weak density-dependence.

DISCUSSION

The two studies provide vital information on the process of decline of these abalone populations. Two effects are conspicuous: the increase in recruitment variability and the spatial contraction of the populations. Increasing recruitment variability implies that a population will be more susceptible to overfishing during years of poor recruitment (Myers & Pepin 1994, Shepherd & Baker 1998). Hence this effect may itself contribute to the spatial contraction of a population because subpopulations with poor recruitment will run a higher risk of increasing depletion, fragmentation and extinction. However, independently of any effect of recruitment variability, spatial contraction may occur through differential recruitment or juvenile mortality throughout the habitat of the original population. We have no data on juvenile mortality but the topographic features defining population boundaries suggest a mechanism for differential recruitment acting at the pre-settlement stage.

Shepherd et al. (1992) noted that topographic coastal or benthic features, such as inlets and rock pinnacles, increased local recruitment strength of greenlip abalone. McShane (1995) reviewed the effects of hydrodynamics on larval transport and concluded that in open waters abalone larvae may be transported large distances but near reef of high relief can be retained near the natal site. Shepherd and Rodda (2001) reviewed other studies showing that islands and bays generate sheer zones and eddies that concentrate larvae. The studies of Shepherd et al. (1992) and Rodda et al. (1997) both suggested transport distances for greenlip larvae of several km from their natal site in places of tidal currents. Studies of the behavior of greenlip larvae (Madigan et al. 1997, Madigan 2000) show that during the trochophore and early veliger stage, together lasting about four days, larvae tend to swim upward. Hence they are likely to be distributed throughout the water column and transported passively in wind-driven and tidal currents for several days before settlement (Sasaki & Shepherd 1995).

Larval behavior at settlement is also important. McShane (1992), McShane (1995), Nash (1992) and Shepherd and Partington (1995) all showed a significant inverse relation between recruitment strength and water movement. McShane (1992) considered that water turbulence could keep larvae away from suitable sites, thus preventing them from making contact with the substratum and responding to settlement cues. Thus several hydrodynamic factors and larval behavior can interact to cause spatial differences in recruitment in abalone habitat.

In greenlip abalone spawning is believed to occur mainly during October to November at around the neap tide (Rodda et al. 1997, Shepherd & Daume 1996, and K. Rodda pers. comm.), so that larvae would remain pelagic to within two days of the beginning of the spring tide when currents are maximal. Thus tidal currents would transport larvae for much of the tidal cycle from neap to flood tide. Hence we may conclude that both in Backstairs Passage and in Avoid Bay, where tidal excursions are at about the same spatial scale as the abalone populations, larvae originating from any subpopulation could settle in any other subpopulation, and are likely to be concentrated by hydrodynamic forces near inlets, headlands and islands. Wind-driven currents and swell could bias up or down any estimated distances of larval transport by tidal currents.

Our recruitment data are consistent with the above predictions. In Backstairs Passage (Fig. 1a), the sites of strongest recruitment, at Lands End, Blowhole Creek, Cooalinga Creek and Spaceship Bay were all in near-shore eddies out of the strong current flowing through the Passage. In Fishery Bay, where recruitment failed earliest, the habitat was of low relief and water currents were very strong. In Avoid Bay (Fig. 1b) populations persisted near the headland of Point Avoid and around Price and Golden Islands, but largely disappeared from the low-relief habitat with stronger tidal currents between the islands. In summary, the three effects, increasing recruitment variability, concentration of larvae by topographic features and an inverse relation between settlement strength and water velocity, can together explain the differential declines observed.

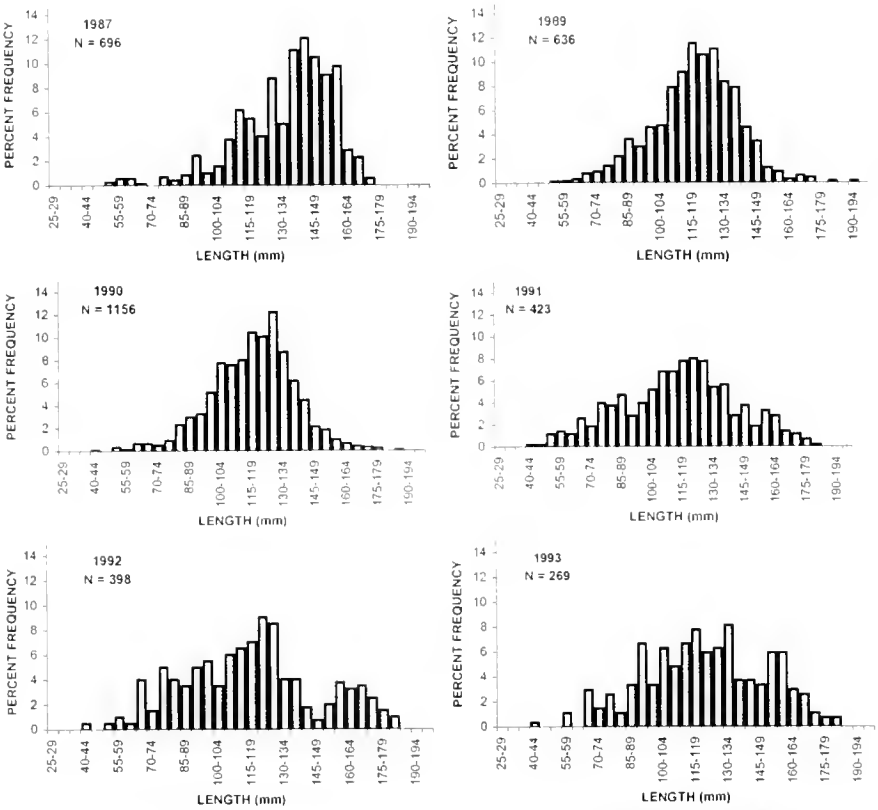

Figure 8. Avoid Bay. Length-frequency data from annual research surveys for all sites combined.

Catch-Abundance Relations

The relation between total catch and abundance is complex and can be confounded by sampling contingencies, spatial contraction of a stock, and diver behavior. We consider them in turn.

Survey densities will vary according to the time of survey in relation to fishing and according to the representativeness of the survey sites in relation to the population response to fishing. Researchers tend to choose sampling sites at shallower, more accessible places close to shore, where subpopulations may be more resilient to fishing and hence may not be representative of the whole population.

Spatial contraction will also confound a direct catch-abundance relation. As abundance is a function of density × area, if the area occupied by the population decreases, overall abundance will decline faster than a decline in density alone at fixed sites, unless the sites are systematically located throughout the spatial extent of the population.

Lastly, divers operate in a visual fishery and a range of human responses may influence their catch. The dominant motivation of commercial divers is to maximize their catch rates (Keesing & Baker 1998, Prince & Hilborn 1998). Divers use their knowledge to fish the largest aggregations, and then progressively smaller ones, and finally cease fishing when densities are low. The decision threshold prompting departure from a reef is principally determined by the catch rate, but other factors such as distance from homeport and depth also play a role (Shepherd & Rodda 2001). Divers accept lower catch rates close to homeport and in shallower water (Keesing & Baker 1998; McShane 1998). Divers also have different spatial strategies, and include: *risk-takers*, divers who fish more reefs and take risks looking for new concentrations of abalone; *followers*, who are content to revisit familiar grounds; and those with intermediate strategies (Prince & Hilborn 1998, McShane 1998). Surveys of South Australian divers by one of us (SAS) have shown that most (~70%) divers tend to fish a group of 5–10 populations with which they are most familiar. But a few, ~20% in the sample, (the risk-takers) show more exploratory behavior and fish up to 20 populations. The remainder has intermediate strategies. The different diver strategies ensure that all exploitable stocks are periodically visited and fished. The implication from these behaviors is that, in the long term, a stable or fluctuating total catch implies stability and a declining total catch, as also declining effort, a declining abundance. While other factors, such as price, weather, and diving conditions, may also affect the behavior of divers there is no evidence that they do other than increase the noise, without changing the underlying relation.

The catch-density curves for the two sites support the hypothesis that the total catch crudely reflects abundance. The relation is noisy and curvilinear as predicted, and suggests that total catch is likely to show hyperstability during the early decline of a population, but when abundance has substantially declined the total catch will decline more steeply.

Stock-Recruitment (SR) Relations

Caddy (1999) noted that in spatially structured populations of sedentary species stock recruitment relations depended on local densities not stock size. Hence SR relations can strictly only be fully described by a family of curves covering every local area with a different recruitment pattern (see Wing et al. 1998, Oren-

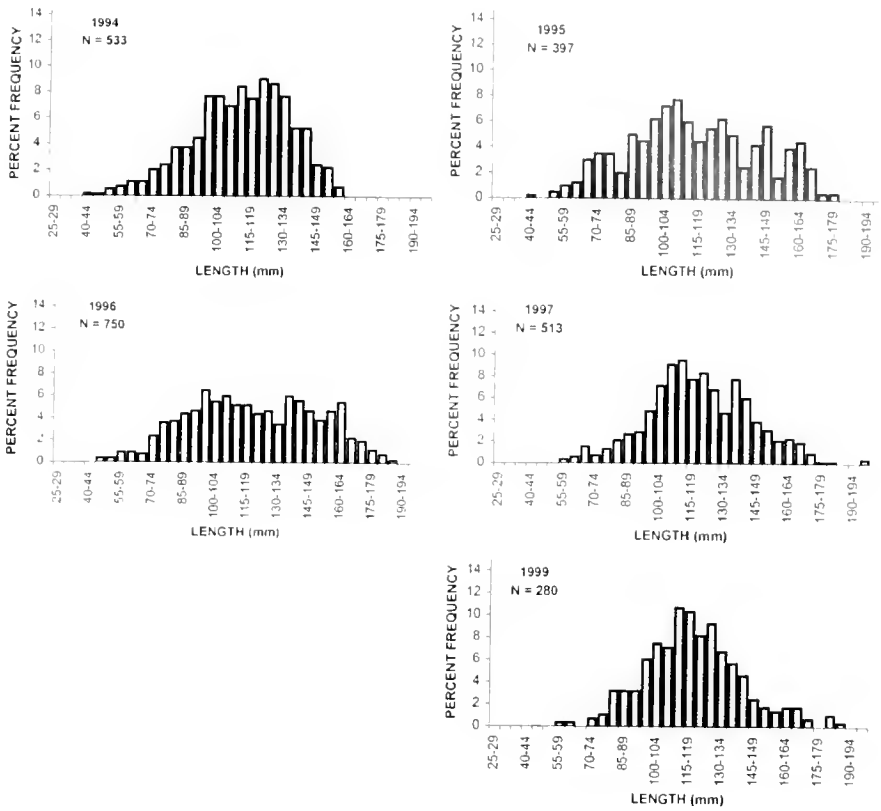

Figure 8. Continued.

sanz & Jamieson 1998 for a discussion of SR patterns in spatially structured stocks). Given the spatial limitations of our data set and the small time series for Avoid Bay, the pair of SR curves presented for each of the two sites can at best be taken as vaguely representing (a) resilient abalone habitats where larvae are retained by hydrodynamic effects, and (b) vulnerable habitats where larval settlement and/or survival are less successful. As discussed previously the models assume a common larval pool.

Whether habitats are vulnerable because of intrinsic factors such as their hydrodynamics or predation, or because of attractant biotic factors, such as the presence of conspecifics, is speculative. On the latter possibility there are four field studies in addition to some laboratory experiments on abalone which suggest that the presence of conspecifics plays an important role in attracting settling larvae. Tutschulte (1976) removed adult abalone from experimental areas of 25 m^2 and found that recruitment in those areas failed. Prince et al. (1987) and Prince et al. (1988) did similar experiments with the same results. Recently Andrew et al. (1998) did the reverse and placed adult abalone in crevices previously without them. They found a 10-fold increase in recruitment. In

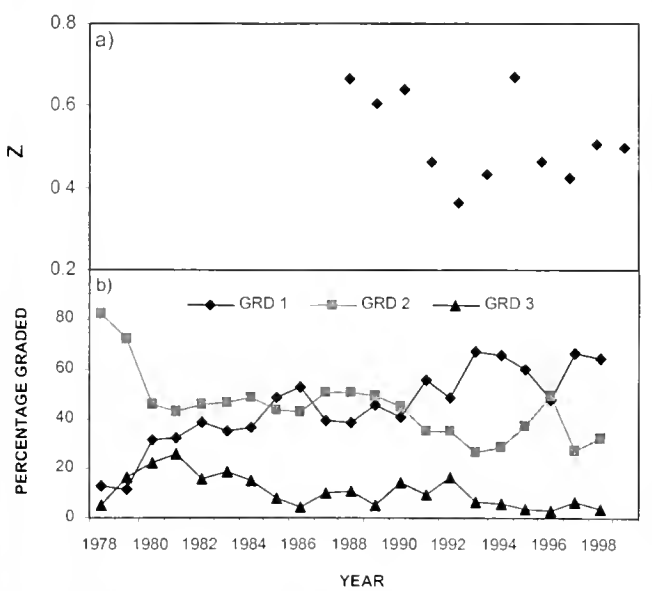

Figure 9. Avoid Bay. (a) change in total mortality coefficient, Z, from 1987–1999; (b) change in proportions of three grades of meat weight in the total catch, expressed as a percentage, from 1978–1998. Grade 1 is < 4 meats/kg; grade 2 is 4–6 meats/kg; grade 3 is >6 meats/kg.

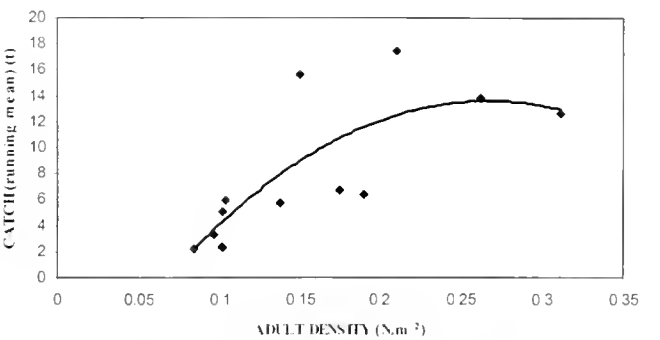

Figure 10. Avoid Bay. Plot of total catch (running mean) vs mean adult density with curve of best fit.

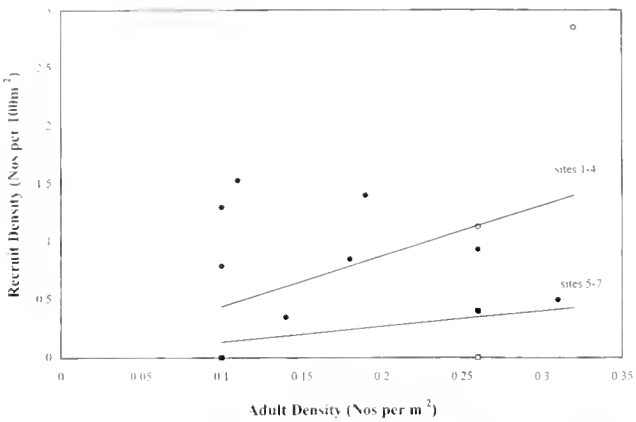

Figure 11. Plots of mean annual recruit density vs adult density for Avoid Bay. Recruit densities are: solid circles for Sites 1–4 and solid squares for sites 5–7 and hollow symbols for years in which adult densities were estimated from the catch. Trend lines for Sites 1–4 and 5–7 are shown. See caption to Figure 6 for 3-y lag time procedure used in the plots.

Western Australia T. Adams (pers. comm.) did a similar experiment with greenlip abalone at a site from which abalone had disappeared some years before and later recorded good recruitment there. Conversely Shepherd et al. (1992) did both additions and removals of adult greenlip at experimental sites with moderate to strong tidal currents but found no effect of local adults (but a strong effect of topography) on recruitment. More recently Daume et al. (1999) showed experimentally that *H. laevigata* settled gregariously in the presence of older conspecifics consistent with the slime trail hypothesis suggested by earlier workers (McShane 1992).

Whatever the mechanism for the failure of recruitment, the SR curves demonstrate the complexities of spatially structured stocks. Unlike the single isolated population in Waterloo Bay with its restricted larval dispersal (Shepherd & Partington 1995), these populations are not internally homogeneous in terms of recruitment strength. Hence SR relations will vary according to habitat and any analysis based on a few survey sites must be a gross over-simplification.

We do not pretend that the SR curves presented here represent the true shape of any underlying SR relation. As argued by Hilborn (1986), Underwood and Fairweather (1989) and Koslow (1992), the concept of a deterministic relation between stock size and recruitment is not easily applicable to invertebrate stocks with high fecundity and correspondingly high, strongly density-dependent mortality during the early life history. Nevertheless, the lower and likely flatter, SR curves for vulnerable habitats at each site suggest the Achilles heel of these populations and the mechanism of their spatial contraction. Flat SR curves imply weak density dependence such that even small increases in exploitation reduces the recruitment rate below the replacement rate and thus leads eventually to population collapse within that habitat (Shepherd & Cushing 1980). Prince and Guzmán del Próo (1993) modeled populations of declining Mexican abalone stocks and found that a similar flat SR relationship best explained the decline of those stocks. Perusal of the SR curves suggest that recruitment overfishing in Backstairs Passage must have occurred about 1980, because no strong recruitments occurred after that year. In Avoid Bay overfishing probably occurred before 1987 when surveys began, but, in the absence of information about the rate of spatial contraction, conclusions about the timing of overfishing events are speculative. Maintenance of productivity of these greenlip populations at the catch levels existing up till about 1985 will thus depend on maintaining abalone densities in vulnerable habitats at much higher levels than those required for resilient habitats.

Fishery Indicators (FI)

The concept of FI and the related one of biological reference points (B.R.P.) have emerged in recent years to enable managers to continuously evaluate the state of fished stocks and employ strategies to prevent their collapse (Myers et al. 1994). The approach is precautionary and accepted both in this fishery (Zacharin 1997) as in international instruments (Caddy 1998, Caddy 1999). Recent developments in this approach include improved decision-making under uncertainty, and consensual adoption of decision rules once limits are approached or exceeded. Nowhere is this more urgent than in abalone fisheries which have depressingly collapsed in nearly all major fisheries in the world (Shepherd & Baker 1998), and now partially in southern Australia (Shepherd & Rodda 2001).

Empirical data from these two stocks provide a testing ground of potential indicators for this fishery. With modification we believe it may be widely applicable to other abalone fisheries. First we note that abalone must be classified as data-poor fisheries, because they comprise many independent stocks for which good fisher-independent data even in the best conditions are seldom available. So what is needed are very simple indices that can be derived 'with only fuzzy knowledge about stock levels and recruitment curves' (May et al. 1978). Such indices are to be sought from (1) historical catch and effort data provided by fishers to government agencies, (2) survey data, and (3) parameters such as the total mortality coefficient, Z, and dependent analyses such as egg-per-recruit (EPR).

Historical Catch and Effort Data

It is almost trite that catch rates (CPUE) offer little or no help as an FI (Breen 1992, McShane 1998, Orensanz et al. 1998). This is because abalones have a clumped distribution, and after fishing they tend to re-aggregate in favored habitats (Shepherd & Partington 1995). Because divers search for aggregations and give up searching at low densities, searching time is not proportional to overall density and catch-rates show hyperstability (Sluczanowski 1986). CPUE thus depends very largely on diver behavior as described previously. Shepherd & Partington (1995) noted that as the fishery in Waterloo Bay collapsed CPUE increased, because divers ceased searching the whole area of the bay and concentrated their effort in the few remnant places where abalone aggregated. A similar behavior may explain the constancy of CPUE in Backstairs Passage (except 1998–1999) and Avoid Bay despite the long-term decline in abundance.

Although CPUE is of dubious value, we propose that a time series of total catch data is a crude but useful indicator of abundance in declining populations, providing it is collected at a fine scale (Shepherd & Brown 1993, Keesing & Baker 1998). The proviso is important because, if the catch data are collected at too coarse a scale, serial depletion occurring at a finer scale is masked. The use of total catch itself is the concept of maximum constant yield (MCY) currently used in New Zealand (Annala 1993, Francis 1993). It is "the maximum constant catch that is estimated to be sustainable, with an acceptable level of risk, at all probable future

levels of biomass". Shepherd and Baker (1998) used the constancy of historic catches for specific metapopulations as empirical evidence on which to base estimates of safe fishing mortality rates (see later in text). Shepherd and Rodda (2001) examined declines in the catch of many other greenlip populations in this fishery and showed that alternative explanations for their decline were so implausible that in this fully exploited fishery, a declining catch should be accepted as evidence of declining productivity.

Changes in the size grading of catches, like other measures based on the size structure, probably reflect changes in the exploitation rate (Andrew et al. 1997, Worthington et al.1998) and may therefore be useful to monitor. However, Keesing et al.(2000) noted great ambiguity in the signal, due to changes in diver behavior and spatial contraction of a stock. The exploitation rate, Z, may remain constant or even fall during the decline of a stock, (Shepherd & Baker 1998), so that neither it nor measures related to size structure are definitive indicators. As in the two examples in this article, populations may collapse with little or no change in CPUE, size grading, length-frequency distributions or Z.

Survey Data

If changes in the spatial extent of declining abalone populations are common, as our data for other declining populations suggest (Shepherd & Rodda 2001), then surveys need to be designed explicitly to cover the spatial extent of the populations to be monitored. If the coverage is partial (as at both sites described here) or biased, then the results are, to that extent, diminished. In research surveys other valuable information such as data on density, the size composition of the emergent population, and the aggregational structure (Shepherd & Partington 1995, McShane 1998) can also be obtained. While spatial information and density estimates together clearly provide the best FI of a stock, cost, especially in deeper water, prevents their widespread or optimal use. Data from Backstairs Passage and Waterloo Bay show a high risk of recruitment failure below mean densities of 0.2 (±0.05)/m^2. The Avoid Bay data suggest even higher densities of ~0.3/m^2 should be maintained, given that the population was already in decline when surveys started. However, a collapse threshold density was only evident for the Waterloo Bay SR curve, possibly due to high larval retention and the more constant recruitment there. The existence of vulnerable habitats, with more variable recruitment, complicates the picture and indicates the need for even higher densities as a buffer against recruitment failure.

Size compositional data are valuable because they can be used to estimate recruitment either in length-based models (Worthington et al. 1998) or in age-based analyses. According to Shepherd et al. (1984) a time series of recruitment is the most unambiguous indicator of abundance in a population. Lastly, there are measures based on the frequency and size of aggregations in surveys. Such measures certainly change as a stock collapses, and they may prove to be valuable, as implied by McShane (1995), and shown by Shepherd and Partington (1995) for the Waterloo Bay population.

Egg-Per-Recruit Analyses and Mortality Coefficients

The problem for precautionary management is to ensure that sufficient spawning biomass is conserved to minimize the risk of recruitment failure. One approach, termed a constant relative escapement strategy, is to set a safe maximum fishing mortality, F (Mace & Sissenwine 1993, Garcia 1996). This leaves a variable spawning biomass from year to year depending on annual recruitment strength. Shepherd and Baker (1998) proposed this for greenlip abalone and suggested $F_{50\%}$ for small stocks and $F_{40\%}$ for large stocks, the difference being due to the observed greater vulnerability of small stocks to overfishing. Here the notation $F_{50\%}$, for example, means that level of F which reduces egg production in an EPR analysis to 50% of the unfished value. This suggestion was based on the analysis of a number of exploited greenlip stocks combined with a global review of nine other abalone fisheries. During the major period of fishing the egg production conserved was 32% in Backstairs passage and 37% in Avoid Bay (O'Loughlin & Shepherd unpublished data). Both were clearly inadequate, and demonstrate the need to reduce F substantially. The recovery of a stock is very unlikely to occur if fishing is permitted to continue in depleted stocks, because divers will continue to fish down the shrinking remnant subpopulation until near extinction. The failure of other collapsed greenlip stocks to recover after 15–20 y (Shepherd & Brown 1993, unpublished data) suggests that depensation at low stock densities may occur.

An alternative strategy is to have a constant absolute escapement, as advocated by Caputi (1992) for a prawn fishery, a strategy that would require routine estimation of annual recruitment strength. This would enable better exploitation of strong year-classes, and may be well suited to short-lived species like prawns with highly variable recruitment. For longer-lived species like abalone in which the catch typically comprises individuals aged six to ten years (Shepherd & Avalos-Borja 1997, Officer 1999), and recruitment strength is very costly to measure, this strategy would confer little advantage and in any event would be practically impossible to implement.

Mace (1994) suggested a threshold biomass based on the shape of the SR relationship i.e., that biomass at which recruitment is one half of the maximum possible. While this is readily determined from the Ricker SR curve for Waterloo Bay (Shepherd & Partington 1995), it is indeterminate for populations like Backstairs Passage and Avoid Bay where SR curves vary spatially and may show a continually ascending curve as in vulnerable habitats. Any chosen threshold from such a curve is dubious because gains in recruitment can always be expected from every increase in spawning stock. Besides, an SR curve will be unknown for all but the very few stocks that have been monitored to collapse.

Other BRPs and FIs are reviewed by Caddy (1998) but either require better data sets than are usually available for abalone or are not appropriate for sedentary stocks.

Use of Multiple Indicators in a Fuzzy System

The above analysis suggests that three kinds of measures, catch data, density, and possibly patch size, data, and $F_{40-50\%}$ values, are the best candidates for use as FIs in the greenlip fishery. They need to be evaluated by simulation and, if shown robust, should then be incorporated into the management protocols described below. Given the lack of information available for individual populations, except for catch data, but the capacity to quickly gain more as required in specific trouble spots, we propose a 'trouble spot thermostat' approach (Caddy 1998, Caddy 1999). First, populations with declining catch (trouble spots) are identified from historic catch data and targeted for assembly of archival information and field survey. Because total catch data tend to be hyperstable early

TABLE 1.

Trouble spot thermostat protocol for decision making after detection of early declines in individual greenlip abalone populations. Read table from bottom to top by analogy to a rise in temperature. The table uses a suite of fishery indicators (left), ordered from levels 1 to 4 according to increasingly severe criteria in a troubled population. Each level leads by an arrow in column 2 to an agreed management response in column 3, and triggers either a demand for more detailed research information or a sterner management response. The ultimate measure is a closure of the troubled population if the criteria are not met.

	Metapopulation Fishery Indicator		Agreed Management Response
4	Total catch decline >60%[3]	→	Close fishery. Establish recovery plan.
	OR		
	Recruitment decline >20% over last 4 years		
3	$F > F_{40\%}$-$F_{60\%}$ according to site[2]	→	Take corrective measures to reduce F (reduced season, closure or increased size limit)
2	Estimated spatial decline >50%	→	
	OR		
	Mean survey density <0.25 m^{-2}		Do EPR analysis. Commence annual recruitment survey.
	OR		
	Z > 0.4		
1	Total catch decline >30%[1]	→	Survey spatial extent of population and compare with historical records. Do catch curve analysis.

[1] Catch decline per map code area since 1985 (Western Zone), 1988 (Central Zone) and 1989 (Southern Zone), being the years in which quotas were introduced.

[2] $F_{40\%}$ for populations of initial productivity >10 t/y^{-1}, $F_{60\%}$ for populations with initial productivity <10 t/y^{-1} (from Shepherd & Baker 1998). Even higher % values of F should be conserved in populations already in serious decline.

[3] Adopt this alternative if corrective measures at level 3 have not been taken; otherwise adopt the second alternative.

in a population decline we propose a small decline (30%) as a trigger for initial action. As the indicators are generated and triggered at level 2 (Table 1), management action is required to commission more data on the trouble spot in question. Then, if the indicator, $F_{40\%}$, for example, is triggered, a more severe management response is dictated. The alternative indicators at several steps reflect the uncertainty inherent in any single indicator. The ultimate response is to close the troubled population to fishing and initiate a stock recovery program.

The consequences of overexploitation of a poorly managed stock are decline and ultimately collapse, so the risks in procrastination are great and will generate large economic losses. A thermostat approach would stimulate timely corrective action, and restore the fishery at a small present cost but with great long-term benefit.

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QUANTIFYING THE PHYSICAL AND BIOLOGICAL ATTRIBUTES OF SUCCESSFUL OCEAN SEEDING SITES FOR FARM-REARED JUVENILE ABALONE (*HALIOTIS MIDAE*)

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ABSTRACT In short term experiments of under 3 mo, carried out in McDougall's Bay, on the northwest coast of South Africa, survival of seeded juvenile abalone (*Haliotis midae*) has been shown to be directly linked to both the size of the abalone at seeding and to the physical attributes of the seeding site. The presence of the sea urchin (*Parechinus angulosus*) has been shown to be insignificant in terms of short-term survival of juvenile *H. midae*. Within the context of selecting the right seeding site attributes, short-term survival was shown to be up to a minimum average of 59% for animals with an average size of 26.61 mm (SD ± 1.33mm), compared to a minimum average of 24% for animals with an average size of 13.87 mm (SD ± 1.73 mm). The fact that the presence of urchins played no significant role in the survival of juvenile abalone, ranging in size from 13 to 27 mm, changes the idea of what the characteristics of successful seeding sites might be. A positive correlation has been established between habitat consisting of stacked boulders, of diameter less than 30 cm, and abalone survival.

KEY WORDS: seeding size, urchin exclusion, site characteristics, habitat, abalone

INTRODUCTION

Seeding experiments using juvenile abalone, *Haliotis midae*, carried out by Sweijd et al. (1998) on the Northwest coast of South Africa showed that some potential existed for the successful introduction (seeding) of this species along this stretch of coastline. To optimize success, it is necessary to understand both the recruitment and ecological dynamics of the species and to determine the life cycle stages that would be most successful in terms of both survival and growth (Tegner & Butler 1989, Davis 1995). One of the most fundamental aspects of the successful recruitment of *H. Midae* is the association between the juveniles of the species and the urchin, *Parechinus angulosus*. This association is considered so strong that the presence of the urchin is considered a driving factor in the population dynamics of this abalone species (Day 1998, Tarr 1995, Tarr et al. 1996). The association between urchin species and abalone species is reported worldwide, and is not restricted to the South African species (Inoue 1987, Kojima 1981, Rogers-Bennet & Pearse 1998, Tegner 2000). Indications are that naturally occurring *H. midae*, up to the average size of 35 mm, are found almost exclusively under urchins (Day 1998, Tarr et al. 1996). While there are also indications that long-term survival of juvenile abalone is linked to the utilization of urchins as refuge, the relationship between artificially seeded *H. midae* and their assumed habitat has not been investigated or quantified. A range of long-term experiments was designed to begin addressing these issues. Initial results, collected over a period of 6 mo, using abalone ranging in size between 8 and 18 mm, showed very low survival rates, ranging from 0 to 12%. This was in contrast to the results of the previous experiment (Sweijd et al. 1998), in which animals ranging in size between 8 and 14 mm showed an average 6-mo survival rate of 30%, seeded in what was regarded as similar seeding environments. While survivors did show a very high association with sea urchins, it became clear that the precise physical attributes of the seeding site, in terms of shelter and protection against both possible predation and against adverse ocean conditions, appeared to be an important factor in survival (Emmett & Jamieson 1988, Schiel 1993). The initial results were confusing; the effects of rough sea conditions could not be differentiated from the effect of abalone seeding size or the influence of the physical or biological attributes of the seeding site. To address these issues, a series of short-term experiments were carried out in McDougall's Bay, Port Nolloth, seventy kilometers south of the Orange River at 29°17′S and 16°52′E. The experimental area is in a bay which, while protected from direct wave action by a reef, is exposed to strong currents and surges. An important advantage of this site was that it allowed constant access by divers to the seeded abalone. The average depth of the experimental area was between 2 and 4 m, with the physical substratum on which the animals were seeded, being predominantly loose, overlapping boulders, of differing sizes. The experiments were designed to address three basic questions:

1. the importance of sea urchins to the survival of seeded juvenile abalone.
2. the influence of abalone size on short-term survival.
3. the type of habitat which offers juveniles short-term protection and shelter.

METHODS

The seeding procedure used for all experiments was the same. The traps used for seeding were those described by Sweijd et al. (1998), consisting of lengths of PVC piping of decreasing diameter, cut in half and placed inside each other. A gap of approximately 1.5 cm was left between pipe sections, and the pipes were then mounted on a flat plastic base. This base was then mounted on a steel plate, which acted as a weight that kept the trap stable on the ocean floor, allowing the animals to leave and return to stable refuge (Schiel 1993). The juvenile abalone were gently brushed into the semi-cylindrical tubes making up the trap. The traps were then closed at both ends with perforated plastic covers that allowed a through-flow of water, and placed into well-aerated, seawater holding tanks. After approximately 30 min, the traps were placed in plastic bags, sealed and placed in Styrofoam containers with sponge-wrapped freezer blocks. The containers were closed and transported to the seeding sites. The trip normally took approximately 20 min. On arrival at the seeding sites, the traps were immediately taken out of the containers and placed into rock

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TABLE 1.

Urchin densities and juvenile abalone survival.

Site	Period (days)	% Survival	Urchins Density Per M^2	% Found Under Urchins	% Found Under Boulders
1	17	5.67	6.79	35.29	64.71
2	17	5.67	0	0	100
3	17	15.67	8.91	27.66	72.34
4	17	3.33	0	0	100
5	23	28.67	0	0	100
6	23	25.33	4.51	39.47	60.53
7	23	28.33	0	0	100
8	23	38	8.59	29.82	70.18

Total number of urchins were counted in a 3 m radius around each trap. Period in days indicates time between seeding and sampling.

pools or in shallow water. The traps were then moved underwater to the seeding sites.

The traps used in the experiment were kept closed until they were in position, and then opened. All the traps were monitored for approximately 20 min after they were opened. Other than a few animals, which were sitting on the plastic covers, no animals exited from the traps and no predators were attracted to the traps in the time immediately following opening. Traps were also observed on a daily basis, and this revealed that by far the greatest majority of animals did not begin to leave the traps until well into the night of the first day, and in many cases not until the second or third day.

Diver efficiency and sighting probabilities were considered. However, due to the fact that only one person was responsible for the searches, the very small numbers of experimental animals used, and the restricted experimental areas, they were disregarded. The sighting probabilities mentioned by Shepherd (1998) were used to weigh the survival figures for the different sized abalone used in the size experiments.

Urchin Exclusion Experiment

To test the short-term effects of the presence of sea urchins on juvenile abalone the following experiment was carried out. Eight experimental sites were selected in typical west coast kelp beds in which there was an abundance of loose boulders, with a biota including local species of crustose corraline algae and sea urchins. The sites were chosen to resemble each other as closely as possible, both in terms of physical appearance and in depth (2–4 m). In a 3-m radius around each trap, an estimate was made of the relative percentage cover of different size boulders and the amount of exposed area between boulders. Percentage cover by encrusting corraline algae was estimated for the experimental area. From four of these sites, all the sea urchins, in a radius of three meters from the trap, were removed. At the other four sites, all those urchins collected from the first four sites were placed directly surrounding the traps. The average density of urchins around each of these traps was 7.20 m^2 (SD ± 2.02). Each trap contained 300 animals with an average size of 12.24 mm (SD ± 1.62 mm) The traps were monitored every few days to make sure that those from which the urchins had been removed remained clear of urchins. The sites were sampled in two groups, after 17 days and 23 days respectively, with each group having two sites with urchins and two sites without urchins (Table 1). On sampling, the number of urchins in a 3 m radius around each trap was counted and the percentage utilization of urchins and boulders by abalone juveniles was calculated for each site, together with abalone survival.

The Effect of Abalone Size at Seeding

The same procedure as above was followed, except that no urchins were removed from any area. Along with percentage cover by encrusting corraline algae (estimated per square meter for the experimental area), urchin densities were calculated around each site. Eight experimental sites were again selected to resemble each other as closely as possible. 200 animals were placed in each trap, 100 larger ones, with an average size of 26.61 mm (SD ± 1.33 mm), and 100 smaller ones, with an average size of 13.87 mm (SD ± 1.73 mm). Placing both the large and small animals together in the traps meant that all the animals were exposed to exactly the same conditions on release. The traps were sampled in two groups of four, the first group after one month, and the second group after two months (Table 2).

Quantifying the Physical Attributes of the Seeding Sites

In an attempt to quantify the physical attributes of each site, a very basic approach was taken. All the experiments mentioned above were treated in the following way. In a 3-m radius around the trap the percentage cover of the surface was estimated according to the following categories:

1. boulders with a diameter of greater than 50 cm.
2. boulders with a diameter of between 30 and 50 cm.
3. boulders with a diameter of less than 30 cm.
4. exposed area, i.e. flat rocky or sandy area that provides little or no protection for juvenile abalone in terms of cracks or crevices that can be used as a microhabitat.

TABLE 2.

Seeding size and juvenile abalone survival.

Site	Period (days)	Size (mm)	Std. Dev (mm)	Survival %	Size (mm)	Std. Dev (mm)	Survival %
1	30	28.07	3.56	62	12.25	1.63	26
2	30	24.64	3.2	55	12.25	1.63	29
3	30	24.64	3.2	68	12.25	1.63	33
4	30	25.53	2.9	54	12.25	1.63	5
5	60	27.5	3.47	66	15.49	2.14	36
6	60	27.5	3.47	59	15.49	2.14	23
7	60	27.5	3.47	50	15.49	2.14	19
8	60	27.5	3.47	33	15.49	2.14	24

One problem with the categories listed above is that boulders are obviously not completely round and while the estimate is carried out on two-dimensional basis, the site is three-dimensional. In an attempt to compensate for this potential problem, the same person did all the estimates and, as a result, there is a degree of consistency throughout the series of experiments. Survival was correlated with the different classifications of site description at the end of each experiment, along with percentage cover of crustose corraline algae species, urchin density, percentage utilization of urchins by abalone per site, abalone seeding size and the ratio between percentage boulder cover to percentage exposed area.

RESULTS

Urchin Exclusion Experiment

At all the sites, sampling was performed around the traps until no more animals were found. During destructive sampling, all boulders were picked up, all the urchins were removed and all crevices and cracks were inspected. The majority of the animals were found within a radius of 2 m of the trap and very rarely were any animals found further than 3 m from the trap. Survival rates give an indication of minimum survival, because some of the surviving animals may not have been found. Destructive sampling can, however, be regarded as effective enough to compare experimental results between sites.

The first four traps were placed on a day in which sea conditions were becoming progressively worse, and the last four traps were seeded during a sustained period of calm sea conditions. This is reflected in the survival rates, with the second batch of animals all surviving in much greater numbers than the first batch. The first four sites were sampled after 17 days and the second four after 23 days. Using the Mann-Whitney U Test, no significant difference was found in the survival of abalone between those sites from which all the urchins had been removed and those sites from which they were still present, $p = 0.67$ (Fig. 1).

The Effect of Abalone Size at Seeding

The first group of four sites was sampled after one month and the second group after two months. Again destructive sampling was used (Table 2). Using the Mann-Whitney U Test, it was found that there was a significant difference in survival between animals that were below 18 mm in length and those that were greater than 24 mm in length ($p = 0.00136$, Fig. 2). This difference in survival

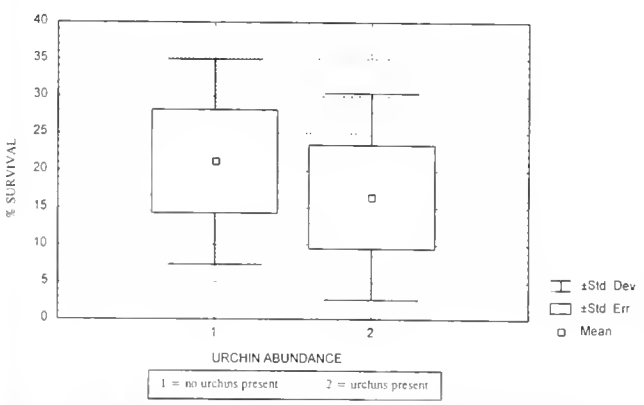

Figure 1. Results from a Mann-Whitney U Test, showing that there is no significant difference in abalone survival between sites with and without urchins.

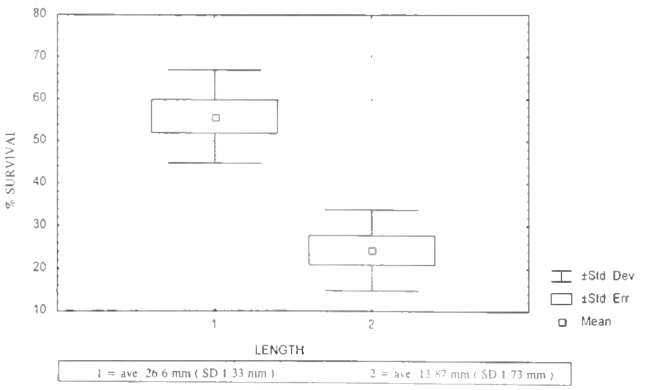

Figure 2. Results from a Mann-Whitney U Test, showing that seeding size makes a significant difference in the survival of juvenile abalone.

was calculated within the context of the similar seeding site characteristics for the different sized animals used in each experiment. While there was a very strong positive correlation between size of seeded abalone and an increase in short-term survival (significant at a 99% confidence level), there was no significant difference between survival after one month and survival after two months, for either size class.

Seeding Site Attribute Classification

Within the context that the substrate is a three dimensional environment, made up of layered boulders and open spaces, the strongest positive correlation (significant at a 95% confidence level) between survival and boulder size is that between survival and boulders with a diameter of smaller than 30 cm. A negative correlation exists between survival and exposed area.

DISCUSSION

The fact that the abalone used in the urchin exclusion experiment were shown to gain no significant benefit in terms of survival, from the presence of urchins, was in direct contrast to expectations (Day 1998, Tarr et al. 1996, Kojima 1981, Scott et al. in prep, Rogers-Bennet & Pearse 1998). These are, however, short term results (Fig. 1). While the advantage, in terms of access to drift kelp, is abundantly clear when observing the masses of drift kelp trapped by urchins, the advantage of the shelter provided by urchins becomes negligible, indicated both by the numbers of unutilized urchins and the extent to which the abalone are hidden in the cracks and crevices provided by rock piles and boulders. Survival rates of juvenile abalone may have been influenced by the fact that abalone not living under urchins would have had to seek other sources of nutrition, thus exposing themselves to predation. Abalone living in close association with urchins have been shown to expose themselves less than animals living in cracks and crevices (Day 1998). Table 3 shows the marginally significant positive correlation between survival and the presence of urchins. The experiments mentioned above, suggest that the juvenile abalone were not relying on urchins for survival. These results are not contradictory to those from the urchin exclusion experiment. This is also reinforced by the insignificant correlation between the percentage of successful abalone that did utilize urchins as refuge. The correlation between the presence of urchins and abalone survival could be interpreted as indicating an area in which there was good habitat for both urchins and abalone (Day 1998). In other words, what is a good habitat for urchins may also be a good habitat for

TABLE 3.

Spearman Rank Order Correlations between site attributes and abalone survival.

Categories	N	R	t(N-2)	p-Level
Boulders with >50 cm diameter	24	0.003089	0.01449	0.988569
Boulders <50 >30 cm diameter	24	−0.109507	−0.51674	0.610492
Boulders with <30 cm diameter	24	0.407414	2.09248	0.048149
Exposed area	24	−0.322266	−1.59675	0.124587
Ratio of boulders over exposed area	24	0.344539	1.72143	0.099207
% Cover corraline algae sp.	24	−0.132326	−0.62617	0.537647
Urchin density	24	0.402841	2.06441	0.050966
Abalone size	24	0.700911	4.60928	0.000136
% Abalone utilizing urchins as refuge	24	0.095773	0.451289	0.656198

abalone. If this is so, the relationship between abalone and urchins may be coincidental.

One of the common features found among the sites discussed above was that the abalone were found clumped in very close proximity to the traps, by far the majority being within a radius of two meters, with as many as 30 or more found under single boulders (Saito 1984, Tegner & Butler 1985). There was very little evidence that the juveniles had moved out of the experimental areas. This is in contrast to results on juveniles of the species *Haliotis rufescens*, which seemed to move very quickly (Rogers-Bennet & Pearse 1998). Observations at seeding sites, specifically chosen to be unsuitable at the preliminary stage of experimentation, did show some abalone staying in the trap for up to 4 mo. In those cases, however, it was obvious that the trap provided the only suitable local shelter. Movement from the trap would obviously expose the juveniles to predation. Not finding the abalone in subsequent sampling would not clarify the question of whether they had moved or whether they had been eaten by predators (Tegner & Butler 1985). The choice of seeding site obviously influences behavior extensively, determining whether an abalone will move and how far it will move (Saito 1984). From the results of the current experiments, it was clear that, if the habitat was suitable, movement was very restricted. Results also showed that the smaller the abalone, the higher the mortality rates resulting from moving into an exposed area. The link between long-term survival of artificially reared juvenile abalone and urchins still needs to be quantified. Whether a habitat of layered boulders could replace any long-term benefit that abalone might derive from the presence of urchins, still needs to be established. This will influence the selection of commercial seeding sites.

In defining the attributes of a successful seeding site, the complexity of a three dimensional habitat made up of layered rocks, and the potential refuge that these may provide, must be kept in mind. The smaller the rocks, the higher the surface area and the more cracks and crevices available per unit area. This may explain the positive correlation between survival and boulders with a diameter less than 30cm, compared to the bigger boulders. While not statistically significant, there did appear to be some type of negative correlation between survival and exposed space. This is to be expected because, in these areas, no shelter was provided for the juvenile abalone. The ratio of area under boulders to area exposed (Table 3), showed a positive correlation with survival (Schiel 1992, Schiel 1993, Shepherd 1998). While this has not proven to be significant, the relationship between sheltered and exposed area was a good, simple measure of habitat suitability. Although the relationship between the amount of crustose corraline algae and survival is shown as not significant in these experiments, naturally occurring juvenile abalone and urchins are found predominantly on encrusting corralines. The movement of sand and general substratum as a result of strong currents is a major and obvious cause of mortality in natural and seeded abalone (Tegner 1992, Tegner 2000). The presence of corraline algae could be an indicator of reef or rock surfaces that are relatively clean of sand, providing abalone with secure footing and space to move, as well as a source of nutrition.

Observations in Mc Dougalls Bay showed the ease with which smaller animals were washed into the water column when boulders were disturbed. Many of the juvenile abalone released themselves without being touched by divers. Many of the sites showed up to 60% losses within one week. Initial mortalities were very high and probably accounted, to a large extent, for much of total mortality. It seems that mortalities in smaller seeded animals may be caused by a lack in the ability to handle strong water currents and disturbances. Farm-reared animals are not exposed to the same extremes of water movement as natural abalone, and animals may need time to adjust to these conditions. It is not clear whether this is a behavioral process or whether it is a muscular strengthening process. Based on this, the size-test experiments were designed (Fig. 2, Table 2). The larger animals used in the experiments showed a completely different response when being sampled. They were much more resilient to being disturbed and, in some cases, removing them caused shell damaged because they were lodged so securely. Increasing seeding size, and thus both the muscle and shell strength of the juveniles, may take pressure off the behavior adaptation phase that the juvenile has to go through after seeding, (Olla et al. 1998, Munro & Bell 1997, Tarr et al. 1996). While there are some differences between the effects of size on survival of seeded *Haliotis rufescens* and *H. fulgens* from the USA (Tegner 2000), the current results on *H. midae*, are similar to those of both Japanese and Australian researchers. While Japanese research has suggested seeding a minimum size of 30 mm, combined with the correct choice of refuge or seeding site for *Haliotis discus* (Masuda & Tsukamoto 1998, Munro & Bell 1997), Australian research has shown that both size and the abundance of predators play an important role in the survival of seeded *Haliotis sp.* (Shepherd 1998). There was also an indication of size playing a role in the experiments of Sweijd et al. (1998). Two different size batches of animals were placed in the same site at two different times. The larger 14-mm animals showed a 39.2% survival compared to a 27.8% survival figure for 8-mm animals, over a six-month period. While the traps were deployed at two different times, resulting in two entirely different sets of environmental conditions acting on the animals, some site attributes would have been the same.

The relationship between seeding size and survival is not a simple one. The effects of size on survival have many facets. Not only does shell thickness increase, but so also does muscle strength. The ability to assimilate survival techniques and experience may also be affected. Seeding is a traumatic event, with high initial mortality. In the case of *H. midae*, it may be possible to address this through correct site selection and seeding size selection. The transition from short-term survival to long-term survival, growth, movement and the environmental factors influencing this need to be investigated. These experiments suggest that differences

exist in the behavior of natural and seeded populations of juvenile abalone. Understanding these differences may lead to a better understanding of the factors that affect survival of seeded juvenile abalone.

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USE OF A SPREADSHEET MODEL TO INVESTIGATE THE DYNAMICS AND THE ECONOMICS OF A SEEDED ABALONE POPULATION

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ABSTRACT Using a simple spreadsheet model it was possible to investigate both the dynamics of a seeded abalone population and the economic implications of seeding such a population. The two variables used to drive the population model were the initial survival after seeding-induced mortality, and age-specific differential mortality. In the model, initial survival or initial population size was the variable that played the dominant role in determining the potential yield of any seeded population. In reality, seeding size plays a significant role in initial survival. Within the same experimental context, 14-mm animals showed a minimum survival of 24% while 26-mm animals showed a minimum of 56%, an exponential increase in potential yield. To address the issue of potential yield on a commercial scale, differential mortality amongst seeded populations of abalone needs to be researched. A cost-benefit analysis has shown that high production costs will only allow ranching to be economically viable in areas where the potential differential mortality regime is very favorable, where survival per age class increases as the population ages.

KEY WORDS: *Haliotis midae*, cost-benefit analysis, commercial seeding, abalone

INTRODUCTION

The possibility of introducing the abalone species *Haliotis midae* to the Northwest coast of South Africa has resulted in the development of an experimental commercial seeding program along a 60 km stretch of coast between Kleinzee and Koingnaas, 120 km south of the Orange River. There are no existing stocks of abalone in this area, though there are fossil records of an extinct abalone species *H. saldanhae*, dating back to more than 1 million years ago (Sweijd et al. 1998). The success of such a commercial venture will be based on a number of criteria, including the following:

1. a very specific understanding of the ecology of the species, especially of the juvenile stage of its lifecycle,
2. the quantification of the mortality of the seeded populations,
3. the long-term economic viability of such a venture.

To do a cost-benefit analysis of the potential project, based on realistic experimental data, (Heppel & Crowder 1998, Hilborn 1998, Schiel 1993), a simple spreadsheet model was used as a means to consolidate biological data collected from seeding experiments. The model was developed after reference to current literature, expert opinion from abalone research scientists and data collected from the present experimental seeding program. As abalone age, the probability of survival to the next age class changes; this is age-specific differential mortality or survival. Experimental data has shown that seeding induced mortality, be it due to handling stress or behavioral adaptation problems, is size related (de Waal & Cook 2001, Schiel 1993, Shepherd et al. 2000). One of the underlying themes of the model was that a trade-off existed between initial mortality and survival of seeded juveniles and the cost of producing juvenile abalone of different size classes. One of the goals in developing the model was to attempt to quantify this relationship. While differential mortality figures do exist for some species (Masuda & Tsukamoto 1998), differential mortality figures do not exist for *Haliotis midae*.

Although these figures are not known, commercial ventures are taking place in South Africa. Being able to explore the ecological limitations in terms of population survival may assist the decision making process and minimize unnecessary risk.

Under natural circumstances, annual recruitment and differential mortality determine population growth. Annual recruitment provides the initial age class every year and differential mortality acts on the population as a whole. In a seeded population of abalone, there is no natural annual recruitment. The seeding number provides the recruits, while the natural process of differential mortality then acts upon the seed. The seeding process is traumatic and initial seeding-induced mortality can be very high (Shepherd et al. 2000, Schiel 1993, de Waal & Cook 2001). Handling and transport, and behavior after release, all play a role in the initial survival of seeded animals. These factors can be addressed, to a certain extent, by improving handling techniques and site selection, and increasing size at release. However, the environmental factors that have a more long-term effect on the differential mortality rates in a population, generally lie outside the realistic scope of human influence. These are also the factors about which the least data exist. When determining total mortality, the impact of initial mortality and differential mortality can be separated from each other to an extent. This can be done by changing the length of the period between seeding and sampling. The shorter the period between release of seed and sampling, the more dominant the effects of seeding induced mortality. The longer the period between seeding and sampling, the stronger the effects of long term differential mortality rates. A period of 30 days has been shown to be long enough for a seeded population of *H. midae* to stabilize after initial seeding-induced mortality. A seeded population that has been in the water for that length of time can be safely assumed to be driven by the same natural environmental factors that affect a natural population in the same habitat.

A model was developed using theoretical differential mortality regimes (Fig. 1a), and experimental initial survival figures for abalone of different sizes (For the purpose of the model, post-seeding survival rates can be interpreted as different initial seeding population sizes). Post seeding survival figures were used as initial population figures in the cost-benefit analysis in this article, and random number generators were used in the sensitivity analysis to initiate populations.

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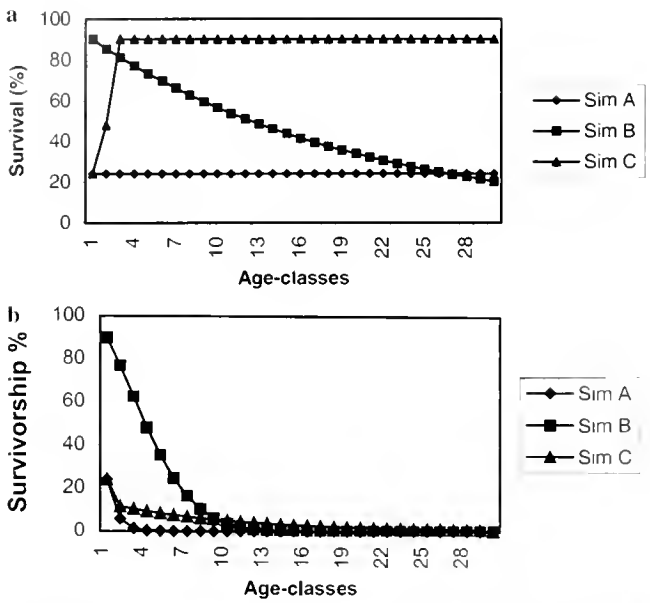

Figure 1. (a) Differential mortality rate regimes used in Simulations A, B, and C. Simulation C is the one selected as being most realistic with survival increasing per age class to a maximum of 90%. (b) Survivorship curves generated by Simulations A, B, and C. These curves show the cumulative effect of differential mortality rates. Although the lines converge over time, the differential mortality rates from Figure 1a were chosen to be completely different. This shows the importance of knowing the actual mortality rates.

METHODS

Description of the Model

To determine population frequency per age class (n) at a specific time period (t), the population frequency in the previous time period (n at t − 1), was multiplied by the age-specific percentage survival figure from the year (t) in the formula:

$$n_t = (n_{t-1}) \times (\%\ \text{survival per age class})_t$$

To determine the percentage survival per age class at a specific time period (t), the percentage survival per age class for the previous time period (t − 1), was multiplied by a factor equal to, greater than or smaller than 1. If the factor is equal to one the percentage survival per age class remains the same for the following year. If the factor is greater than one, survival per age class increases per age class. In other words, the older an animal becomes the less chance it has of dying. If the factor is less than one, survival per age class decreases and the percentage chance of dying increases with age.

Age class frequencies were calculated as an average at the end of a cycle (one run), which for simulation purposes could be up to 30 y, the maximum life span for *H. midae* (Tarr 1995). Age class periods were equal in length of time to the first time period. All simulations in this case were done in time periods of 1 y.

The following are assumptions made to develop the model.

1. Differential mortality rates were developed using the assumption that percentage mortality per age-class either increased or decreased by a constant factor in consecutive age classes (Fig. 1a).
2. Differential mortality rates included all limiting factors on survival, density dependence, senescence, carrying capacities etc.
3. Maximum survivorship of any age class could be 90% of the previous age class. (Average annual mortality is taken to be approximately 11% for all size classes of natural abalone stocks by the Marine and Coastal Management Institute of South Africa (pers. com. C. Moloney).
4. There was no natural recruitment taking place in the seeded population.

Investigating the Range of Potential Differential Mortality Regimes

While a range of differential mortality rates may exist for any species in a range of habitats, it was necessary to restrict the investigation to only three experimental options. In the following simulations, an initial survival figure of 24% was used.

Simulation A

A basic approach to modeling a differential mortality regime is to assume a constant mortality rate per age class throughout the entire population, regardless of age. This is a simplistic approach and there are no indications that *H. midae* does behave in this way (Fig. 1a).

Simulation B

In this case, it was assumed that mortality increased per age class by 5% per year from a maximum survival rate of 90%. However unrealistic, this allowed a broader investigation into the possible survival options and behavior of the model (Fig. 1a).

Simulation C

In this case it was assumed that mortality decreased per age class, survival per age class doubled to a maximum of 90% (Fig. 1a). The shape of this survival curve seems to be the most realistic option, although the exact age or size specific differential mortality rates and the gradient of the curve are not known. (Tegner 2000, de Waal & Cook 2001, Shepherd et al. 2000, Schiel 1993).

Figure 1b shows survivorship per age class generated by the percentage survival per age class curves from Figure 1a and the initial population number of 240,000 (based on a 24% post seeding survival figure). Figure 1b shows the cumulative survival percentage or the probability of an animal reaching a certain age. This was the figure that indicates the yield per recruit of a seeding operation in terms of age class frequency distributions.

Sensitivity Analysis of the Model with Regards to Survival Regime and Initial Survival

It was necessary to test the behavior of the model in relation to the two driving variables, seeding induced mortality and differential mortality. To do this, the model was run with stochastic recruitment generated at the beginning of each year. Two hundred runs were done for each scenario analysis. A random number generator between 1000 and 100 million was used to simulate recruitment at the beginning of every year.

Four general scenarios were developed to test the sensitivity of the model to the two variables (Fig 2). In Group 1, differential mortality rates were manipulated over time while initial survival figures, or initial population sizes, were kept constant. In Group 2 differential mortality rates were kept constant while initial population sizes were manipulated over time.

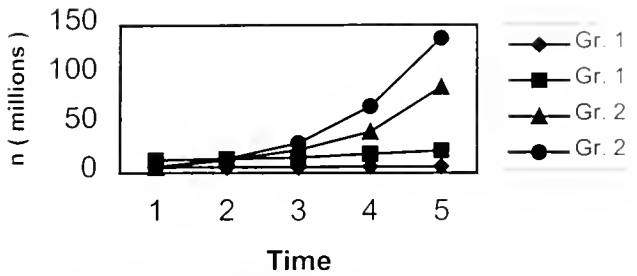

Figure 2. Sensitivity analysis results in which either initial population numbers were manipulated while differential mortality rates per age class were kept constant over time, Group 2. Alternatively, differential mortality rates were manipulated while initial population size was kept constant, Group 1. The period of time is conceptual and is only used to show the difference in total population numbers as a result of manipulating the two driving variables.

Cost-Benefit Analysis

A simple cost-benefit analysis was developed using experimental initial survival figures for both 14-mm and 26-mm seed sizes, a range of differential mortality regimes, and the present market price for abalone. A purchase price of R 1 per animal for 14-mm animals and R 4 per animal for 26-mm animals, and a selling price of R210 per kg for 300 g abalone, were assumed. The exchange rate used was R 6 (SA) to $1 (US). Prices were quoted by Port Nolloth Sea Farms.

A series of differential mortality rates were used in which survival per age class ranged from being constant to increasing by 60% per age class as the population ages. Initial population figures were based on experimental data (de Waal & Cook 2001), 24% for animals with an average size of 14/mm, and 56% for animals of average size 26/mm. One initial seeding batch was allowed to run the course of the model to a market size of 300 g. Using an average growth rate of 1.5 mm per month, and a mass-length curve measured from animals that have been at sea at Kleinzee for 13 mo, this size would have been reached after approximately 6.5 y (unpublished data).

RESULTS

Sensitivity Analysis

Both driving variables, seeding induced mortality and differential mortality, had an influence on the model output in terms of the rate of population decrease. Over the same time period, in those simulations in which initial population size was manipulated, Group 2 (Fig. 2), the proportional impact was far greater than in those simulations in which differential mortality rates were manipulated, Group 1 (Fig. 2). While model output was generated by a combination of the effects of both variables, it is clear that initial survival after seeding-induced mortality had stabilized, played the dominant role.

Cost-Benefit Analysis

With an average growth rate in the range of 1.5 mm per mo measured in Port Nolloth for farm-reared juveniles, it would take approximately 9.5 mo for animals to reach 14/mm, and a further 7.5 mo to reach 26 mm, a total of 17 mo. The animals seeded at size 14 mm would reach harvesting size after 6 y at sea. The animals seeded at 26 mm reached that size after 5 y. On average however, harvesting for both seeding scenarios could take place in the seventh year after spawning. Growth rates are variable and this must be kept in mind when interpreting analyses. For example, the estimated time to legal size for experimental animals seeded at Port Nolloth was estimated to be anywhere between 3.5 y and 6 y (Sweijd et al. 1998).

DISCUSSION

The model was designed to investigate the dynamics of a single population of abalone, based on contemporary knowledge of the dynamics of abalone in general. The ultimate goal would be to generate experimental data to the point where modeling can be based on accurate differential mortality and initial survival figures. At this stage, this was not possible. However, a choice based on scientific facts could be made between realistic and unrealistic options. Indications from this project, and from contemporary literature, are that differential mortality decreases with age, i.e. survival increases until senescence sets in. Davis (1995) estimated survival of juveniles in artificial habitats to be 32% after 1 y and 24% after 2 y. In other words, in the second year survival had increased to 75% compared to 32% in the first y. Seeding-induced mortality would account for a large percentage of the first y mortality, and differential mortality would account for the decrease in mortality of the seeded population in the second year (Shepherd et al. 2000). Seeding-induced mortality is clearly the most important factor to consider when seeding.

The percentage survival and the survivorship curves, in Figures 1a and 1b, showed very clearly that it is quite possible for theoretical curves to converge at some point. If only one point value is known, very little could be determined about the dynamics of the model. To model production accurately, it would be necessary to have a good idea of the differential mortality regime acting in that specific habitat. The goal of objective research should, in this case, be to determine the most realistic mortality regimes in any given situation. Models used with appropriate experimental data will allow analysis of the feasibility of projects before any serious financial input is made and should be used and developed as more biological data become available (Munro & Bell 1997, Wilson et al. 1998, Heppel & Crowder 1998, Hilborn 1998). It is absolutely essential to understand the concept of differential mortality and survival before any predictions can be made about the success of seeding programs.

The model showed that, with the quoted purchase price of seed, it is not economically viable to seed juvenile abalone of either size if the survival per age class does not increase by at least 10% per age class. If the survival per age class increases by more than 40% per age class, it would be viable to seed even the smaller animals. The model showed very clearly that there was an exponential increase in potential yield for larger seed animals compared to smaller seed animals (Table 1). This is a reflection of what happens under natural conditions (Shepherd at al. 2000, Schiel 1993, Tegner 2000). Experimental results by De Waal & Cook (2001) have shown an exponential increase in survival of juvenile abalone with an increase in seeding size. This could possibly be attributed to an increase in muscle and shell strength of the juvenile *H. midae*. Both behavior and the ability to adapt could also play an important role in this differential survival. According to the model, this difference in potential yield was caused by both the strong effect that initial survival has on the model output, and by the speed at which high initial survival causes the survival per age class value to reach a maximum of 90%. It is important to note that while experimental evidence suggests (unpublished data) that both

TABLE 1.

Cost-benefit results for abalone harvested after approximately 6.5 years at sea.

Survival Per Age Class	Seeding Size mm	Harvesting Mass kg	Buying R (mill)	Selling R (mill)	Profit R (mill)
Remains constant	14.00	13.76	1.00	0.00	−1.00
	26.00	9252.30	4.00	1.94	−2.06
Increases by 10%	14.00	101.81	1.00	0.02	−0.98
	26.00	38568.32	4.00	8.10	4.10
Increases by 20%	14.00	633.00	1.00	0.13	−0.87
	26.00	80243.65	4.00	16.85	12.85
Increases by 30%	14.00	2641.08	1.00	0.55	−0.45
	26.00	86416.24	4.00	18.15	14.15
Increases by 40%	14.00	5463.39	1.00	1.15	0.15
	26.00	92588.83	4.00	19.44	15.44
Increases by 50%	14.00	8264.97	1.00	1.74	0.74
	26.00	98761.42	4.00	20.74	16.74
Increases by 60%	14.00	11145.12	1.00	2.34	1.34
	26.00	99202.32	4.00	20.83	16.83

adults and juveniles may survive well in the same habitat, this may not always be the case. Seeding must be done in an area in which both initial mortality and long term mortality are as low as possible.

In any potential ranching and stock enhancement project, production costs that are too high do not allow continuation. This model has allowed an insight into the limitations set by natural processes before the production process has begun (Hilborn 1998, Moksness et al. 1998, Schiel 1993). Production costs could be much lower than the quoted purchase prices and better potential income could be obtained by producing low-cost abalone seed rather than purchasing it. A trade-off exists, not only between supply and demand in terms of the market price of abalone, but also between market price and the seeding strategy and site characteristics of the project concerned. In abalone ranching, production costs must be seen to include both the negative effect of initial seeding mortality and the negative effect of the potential differential mortality rates that exist. The importance that these two variables have on the potential economic viability of a ranching project, and the lack of experimental data concerning these variables, emphasizes the need for additional research in this field.

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TRANSMISSION OF THE RICKETTSIALES-LIKE PROKARYOTE "*CANDIDATUS XENOHALIOTIS CALIFORNIENSIS*" AND ITS ROLE IN WITHERING SYNDROME OF CALIFORNIA ABALONE, *HALIOTIS* SPP.

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ABSTRACT Withering syndrome (WS) is a chronic, wasting disease responsible for mass mortality in southern California populations of black abalone *Haliotis cracherodii* and responsible for significant losses of cultured red abalone *H. rufescens*. Ongoing studies in our laboratory indicate that a recently described gastrointestinal Rickettsiales-like prokaryote, "*Candidatus Xenohaliotis californiensis*" (RLP) is the etiologic agent of WS. Here we describe attempts to experimentally transmit the RLP and demonstrate its role in WS. In two preliminary experiments, RLP-infected black abalone postesophagus homogenate (PEH) was injected into the foot or orally administered to RLP-free black abalone. No RLPs were detected 8 wk after pedal injection. Low rates of transmission were observed 8 to 12 wk after oral inoculation, although an RLP-positive animal was also detected in a negative control group inoculated with filtered seawater. In a separate, 16-wk study, RLP infections were detected in red abalone that received effluent from a tank of infected red abalone while control animals that received direct-source seawater remained RLP-free. A fourth, long-term study delivered PEH or seawater to RLP-free red abalone with either bath exposure or intra-digestive gland injection. An additional treatment to test a potential viral etiology for WS consisted of intra-digestive gland injection with a 0.1–0.2 μm filtrate of PEH and subsequent treatment with antibiotics. Each treatment was administered six times over a 16-wk period. Cohabiting RLP-infected black abalone with RLP-free red abalone provided a positive control treatment. At wk 63 post-initiation, untreated, saline injected and PEH filtrate injected groups had low cumulative mortality (0–20%), while mortality in the PEH bath, PEH injection and cohabitation treatment groups was 70–90%. There were statistically significant relationships between experimental treatment, RLP burdens and signs of WS. Low-level RLP infections of uncertain origin were observed in one each of the duplicate tanks of the negative control and the PEH filtrate-injected animals. An absence of WS signs in recipients of the PEH filtrate provides strong evidence that the agent of WS is non-viral. Collectively these studies provide solid evidence that the RLP is the etiologic agent of WS.

KEY WORDS: *Haliotis rufescens, cracherodii*, Rickettsiales, withering syndrome, transmission, abalone

INTRODUCTION

The black abalone *Haliotis cracherodii* is one of several haliotid species found on the Channel Islands and mainland of southern and central California, USA. This species lives in the intertidal zone and is considered less desirable for human consumption than several subtidal species, but became an important component of the commercial fishery in the 1970s when stocks of subtidal species declined, presumably due to increasing recreational and commercial fishing pressures (Davis et al. 1992). Resource managers and abalone fishermen first noticed large numbers of dead and dying black abalone on the Channel Islands in the mid-1980s (Haaker et al. 1992). The term Withering Syndrome was coined to describe the affected abalone, characterized by lethargy and a greatly reduced pedal mass (Haaker et al. 1992). Southern California black abalone populations have essentially collapsed, with mortality greater than 90% in many areas (Haaker et al. 1992, Richards & Davis 1993). Stocks of subtidal red (*H. rufescens*) and pink (*H. corrugata*) abalone also declined during the 1980s (Davis et al. 1992) but the role of fishing pressure, climatic events and WS in those declines remains unclear.

Although the appearance and severity of WS outbreaks appeared to be enhanced by warm water temperatures (Steinbeck et al. 1992, Tissot 1995), the epizootiology of the disease indicated involvement of an infectious agent (Lafferty & Kuris 1993). WS spread throughout the Channel Islands followed by movement to the California coastline, where it advanced northward during the early 1990s (Lafferty & Kuris 1993, Alstatt et al. 1996). An initial investigation of nine black abalone with WS and five without signs of the disease reported the presence of several parasites and a gastrointestinal Rickettsiales-like prokaryote (RLP), although none occurred in all affected abalone while being absent in healthy-appearing abalone (VanBlaricom et al. 1993). The RLP formed large intracellular inclusions in gut epithelium, causing hypertrophy but little other cytopathology. RLPs are common endosymbionts in molluscs, often being unassociated with disease (Sparks 1985, Elston 1986). Gardner et al. (1995) examined black abalone from both where WS occurred and where it did not. They found that the RLP was present only in abalone from the WS-endemic site, and noted pathological changes in the digestive gland that could be due to the RLP. Subsequently, Friedman et al. (1997) examined starvation, temperature, the renal coccidian *Pseudoklossia haliotis* and the gastrointestinal RLP as causes of WS. While eliminating the coccidian, starvation and elevated temperature as being directly responsible for WS, complex relationships between the RLP and temperature warranted further investigation. Friedman et al. (this volume) transmitted WS and the RLP between black abalone. Groups of initially RLP-free black abalone that contracted the RLP by cohabitation with RLP-positive animals had a higher proportion of animals with signs of WS and higher proportion that died than did unexposed RLP-free control animals. However, the authors also observed a lack of significant correlations between WS signs and RLP infection intensity, further indicating a complex relationship in black abalone.

The RLP is not restricted in host species to the black abalone.

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The RLP is present in most or all abalone aquaculture facilities in California, which raise red abalone to a market size of approximately 9 cm in land and cage-based operations throughout the state (McBride 1998). This is due to its increasing range in wild populations and the widespread distribution of infected seedstock prior to it being recognized as a potentially significant pathogen (C. S. Friedman, unpublished observations). During the 1997–1998 El Nino, red abalone aquaculturists in the southern and central portions of the state began observing sharply elevated frequencies of animals showing WS signs in association with elevated water temperatures. Recently, Moore et al. (2000) demonstrated that farmed red abalone with RLPs can show little or no signs of WS at lower temperatures (14.7°C), but elevation of temperature to 18.5 C caused elevated mortality, expression of WS signs and increased RLP burdens. With increasing evidence for its role in WS, Friedman et al. (2000) recently provided a description of the RLP, designated "*Candidatus Xenohaliotis californiensis*." The term "*Candidatus*" in the taxon indicates that the species was described largely on morphological and DNA sequence-based data and still requires the serological and biochemical analyses that would be performed if the species could be cultured *in vitro*.

The coinciding geographic boundaries and correlation between WS and the RLP do not alone demonstrate that this agent causes the disease. An alternative hypothesis suggests that the RLP proliferates in host animals debilitated by WS, itself caused by an unidentified, possibly viral agent. Our current studies, while limited by the difficulty of obtaining and maintaining RLP-free abalone, were designed to experimentally transmit the RLP and examine links between severity of RLP infection and expression of WS signs.

MATERIALS AND METHODS

The experimental designs of the four consecutive experiments conducted are shown in Figure 1. Experiments 1–3 were designed to investigate transmission of the RLP. Animals were therefore sacrificed at time periods of several weeks to several months, generally before expression of WS occurred. Experiment 4 was designed to allow sufficient time for development of WS and was terminated following extensive mortality in treatment groups 15 mo post-initiation.

Animals and Animal Husbandry

All experiments were conducted in the Pathogen Containment Facility at the Bodega Marine Laboratory, from which all effluent is treated with 10 mg/L chlorine for two hours and dechlorinated with sulfur dioxide before release. Lidded, 8-L tanks with outflows situated so that each tank held 5 L were used in Experiments 1–3. In Experiment 4, lidded 58-L tanks with standpipes situated to hold 50 L were used. All tanks received sand-filtered, aerated seawater and were supplied with kelp (*Macrocystis pyrifera*) several times per month. Care was taken to prevent cross contamination between tanks, including submersing gloved hands, measuring tools etc. in a tamed iodine solution (Prepodyne) and spraying surfaces with 70% ethanol as necessary. Animals were tagged by inserting either a numbered stainless steel washer on stainless steel wire or a numbered vinyl sleeve (Floy Tag, Seattle) on a cable tie through the first and second most recently formed respiratory pores. Benzocaine (Sigma, 40 mg/L, 15 min baths) was used as an anesthetic to dislodge animals for transfer or treatment.

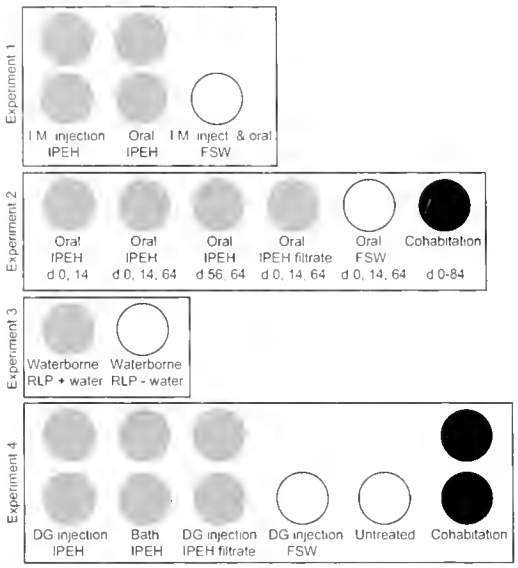

Figure 1. Schematic diagrams of the experimental units in Experiments 1 through 4. Circles represent the numbers of tanks in each treatment group, with experimental tanks shaded, negative control tanks white and positive control tanks black. The first line of text indicates the method of delivery of the material shown in the second line of text. For Experiment 2 the third line of text shows the days on which animals were treated; the first group was sacrificed at day 28 and the remainder on day 84. IPEH: RLP-infected black abalone postesophagus homogenate; FSW: Filtered seawater; DG: Digestive gland.

Histology: Quantification of RLP Infection Intensity and WS Signs

Animals that died during each experiment and all survivors at experiment termination were processed for histological detection of RLP burden. Animals in Experiment 4 were also assessed for WS-associated pathomorphological changes in the digestive gland and foot. Tissue sampling and histological processing was performed as previously described (Moore et al. 2000). Davidson's-fixed (Shaw & Battle 1957), hematoxylin and eosin-stained 5 μm paraffin tissue sections containing postesophagus, digestive gland, foot muscle, kidney and gonad were prepared for each animal, and slides were encoded to prevent bias during assessment. RLP infection intensity was estimated for the postesophagus and digestive gland (the two tissues in which RLPs can be found at high densities, Friedman et al. 2000). RLP burdens were quantified in each tissue using the scale of Friedman et al. (1997) based on the average number of RLP inclusions per 200× magnification field of view: absent = (0), 1–10 = (1), 11–100 = (2), or greater than 100 = (3). Four different disease signs of withering syndrome were assessed using integral scales from 0–3, modified from Moore et al. (2000). For all four parameters, (0) represented a normal healthy appearance. For body shrinkage, (1), (2) and (3) represented slight, moderate and severe shrinkage respectively; for digestive gland metaplasia or digestive gland atrophy, (1), (2) and (3) denote 5%–10%, 11%–25%, and greater than 25% of the digestive gland being comprised of transport duct epithelia or connective tissue, respectively. Foot degeneration scores of (1), (2) and (3) denote muscle fibers comprising 76%–90%, 51%–75% and less than 50% of the foot muscle, respectively. A condition index, CI = total weight, g/(shell length, cm)^3, was also used to assess body shrinkage. The presence of the renal coccidian *Pseudoklossia haliotis* (Friedman et al. 1995) was also noted.

Preparation of Infected Postesophagus Homogenate (IPEH)

Experiments 1, 2 and 4 attempted transmission of the WS-RLP using several methods of delivering homogenized, RLP-infected postesophagus tissue excised from adult black abalone (IPEH). Black abalone donor animals were collected from Vandenberg Air Force Base, California, where WS has been endemic since approximately January 1994 and RLP prevalence was approximately 90% during the collection period (C. S. Friedman & J. D. Moore, unpublished observations). Animals were collected several times per year during 1996–1998, held in 9–13°C seawater and fed kelp *ab libitum*. Postesophageal tissue (including the "crop" as described by Bevelender, 1988) from three to eight animals was pooled and minced in 0.2 μm filtered seawater (FSW) on ice. The tissue was homogenized with a 7-ml Tenbroeck homogenizer. The tissue homogenate contained large pieces of connective tissue and other debris which were removed by gentle centrifugation (100-250 × *g* for 2–5 min). The clarified homogenate was transferred to a new tube and diluted with FSW as necessary to obtain required volumes as described below.

Experiment 1: RLP Transmission by Intramuscular Injection or Oral Inoculation

In July 1997, black abalone were collected from Sobranes Point (Carmel), California, a location where WS had not been observed. One month after collection, the animals were randomly distributed to 5 tanks (4–5 abalone per tank) and acclimated from 14°C to 19°C over 1 wk. The treatment groups are shown in Figure 1. The IPEH consisted of 1.745 g of postesophagus from four black abalone diluted to 15 ml. The IPEH was administered at a rate of 6.8 μl IPEH/ g whole animal weight to four animals in each of two duplicate tanks by either intramuscular injection in the foot with a 23-gauge needle or oral delivery using a 23 gauge animal feeding tube inserted into the mouth. A single tank (n = 5 animals) served as a negative control; animals were treated with both oral delivery and intramuscular injection of FSW. All animals were sacrificed on day 60 post-initiation and examined for the presence of RLPs by histology.

Experiment 2: RLP Transmission by Oral Inoculation

In August 1997, black abalone were collected from Carmel Point (Carmel), California, where WS had not been observed. In March 1998 the abalone were randomly distributed into six tanks (n = 6 animals/tank). Temperatures during the experiment ranged from 11.5°C–15°C until day 40, after which temperatures were elevated to 17°C–20°C, with an overall mean ± s.d. of 15.6°C ± 2.8°C. Treatments (Fig. 1) consisted of multiple oral inoculations with IPEH or a 0.2 μm filtrate of IPEH at a rate of 3.0 μl/g whole animal weight on days 0, 14 and 64 post-initiation. The IPEH on each day of treatment consisted of 5.2–7.5 g of postesophagus from three or four black abalone brought to a volume of 15 ml with FSW. To prepare IPEH filtrate, IPEH was centrifuged (400 × *g*, 5 min) and then filtered twice through 0.2 μm syringe filters. As a positive control, three black abalone from Vandenberg Air Force Base were added to one tank of the Carmel Point abalone and maintained until termination of the experiment. On day 56, after discovering negative results in the IPEH treated group that was sacrificed at day 28, a previously untreated group was inoculated with a more concentrated IPEH (2.8 g postesophagus in 5 ml FSW without centrifugation). One IPEH treated group was sacrificed at day 28 and all other groups were sacrificed on day 84 post-initiation and examined for the presence of RLPs by histology.

Experiment 3: Waterborne RLP transmission

In March 1998, adult red abalone were collected by scuba from Caspar Cove and Mill Cove, Mendocino County, California where WS had not been observed. In August 1998, 14 animals were divided equally into two groups and placed in 8-L tanks. Each of the two tanks was connected to its own intermediate 8-L tank by approximately 25 cm of tubing. The inflow to the intermediate tank of one treatment was connected to an 18°C water source (= control). The inflow to the other intermediate tank was connected to the outflow of a 30-L tank that contained approximately 60 RLP-positive red abalone 2–10 cm in length and was supplied with the same seawater source. The animals were sacrificed 111 days post-initiation and examined histologically for the presence of RLPs.

Experiment 4: WS Development Following RLP Transmission by Digestive Gland Injection or Bath Exposure

Experiment 4 utilized animals from the same collection as Experiment 3. In July 1998 one hundred animals were tagged, equally distributed among ten tanks and allowed to acclimate for 29 days, during which period the water temperature was raised from 15°C to 18°C. Animals were treated on days 0, 21, 35, 56, 91 and 109 of the study with the treatments shown in Figure 1. On each treatment date, IPEH was prepared from 6.5–7.9 g of postesophagus tissue excised from six to eight black abalone and brought to a final volume of 160 mL with FSW (FSW/3% heat-inactivated fetal bovine serum on day 0). The IPEH filtrate was prepared by successive filtration of IPEH through 8 μm, 0.8 μm, 0.2 μm and 0.1 μm filters, except on day 0, when 0.1 μm filters were unavailable and the 0.8 μm filtrate was put through 0.2 μm filters twice. For all digestive gland injection treatments (FSW, IPEH, IPEH filtrate), animals were injected with 3.6 μL/g whole animal weight in the posterior ventral portion of the digestive gland using a 23 gauge needle. Recipient animals ranged in weight from 221 g to 1,360 g resulting in injection volumes ranging from 0.8 ml to 4.9 ml. For the bath treatment, tanks were drained and brought to a volume of 30 L, to which 25 mL of IPEH was added. Tanks were then maintained statically with aeration for two hours, after which flowing seawater was returned. The cohabitation positive control replicates were each supplied on day 0 with three Vandenberg black abalone showing variable degrees of body shrinkage. Black abalone that died were replaced until day 104, at which time all black abalone were removed.

After the discovery that an animal in the IPEH filtrate treatment that died on day 91 was infected with the RLP, a decision was made to inject these animals with antibiotics. This was based on the design of this treatment to allow a potential viral pathogen to infect recipients in the absence of the RLP. These abalone were injected in the foot with oxytetracycline (10 mg/ml in 2% saline) at a rate of 21 mg/kg tissue weight. Injections were made every 48 h for a total of three injections, followed by two to three weeks without injections and repeating the series two more times for a total of nine injections over 47 days.

Five feeding trials were conducted to measure the amount of kelp consumed by abalone in each treatment. An amount of kelp equal to 15% of the total body weight in each tank was added with the amount remaining after 24 h being reclaimed and weighed.

The experiment was terminated on day 446 post-initiation when mortality in the bath treatment approached levels seen in the cohabitation and IPEH injected groups. Statistical analysis of the results from Experiment 4 included a Chi Square contingency table analysis comparing IPEH injected, IPEH bath, cohabitation and IPEH filtrate-injected treatment groups to the negative control group ($\alpha = 0.05$). Data for WS signs (body shrinkage, digestive gland metaplasia, digestive gland atrophy or foot degeneration) were condensed into low (score of 0–1) or high (score of 2–3) categories of severity and 2 × 2 contingency tables compared observed vs. expected frequencies in each treatment group and the negative control group. Similarly, frequencies of low vs. high levels of each WS sign were compared in animals with low-level RLP infections (RLP intensity score 0–1) vs. high level infections (RLP intensity score 2–3) for the postesophagus and digestive gland separately. When the frequency in any cell was less than five observations a Fisher Exact test was employed.

RESULTS

Experiments 1 and 2: RLP Transmission by Intramuscular Injection or Oral Inoculation

In Experiment 1, 60 days after a single injection or oral inoculation with infected black abalone postesophagus homogenate (IPEH) or filtered seawater, one individual that received IPEH orally contained a single focus of two RLP inclusions in the postesophagus (RLP intensity = 1). In Experiment 2, no RLPs were seen among the six animals sacrificed at day 28. Upon termination at day 84, RLPs were detected in postesophagi of two of the six individuals orally inoculated with non-clarified IPEH on days 56 and 64, and in one individual in the FSW-injected negative control group. The RLP inclusions in these animals were small and very infrequent (RLP intensity = 1). Five of the six cohabitation positive control animals had low RLP intensity scores (RLP intensity = 1 in postesophagus, digestive gland, or both) and in one animal no RLPs were detected.

Experiment 3: Waterborne RLP Transmission

Histological analysis of survivors in each tank at day 111 revealed the RLP to be present in 100% (6/6) of the animals receiving effluent from the RLP-positive tank, while those receiving direct-source seawater had no RLP infections (0/6). One animal in each tank died prior to termination and in each case tissues were too necrotic for histological analysis. Intensities of postesophagus and digestive gland infections in the six RLP-positive animals ranged from relatively mild (1) to severe (3).

Experiment 4: WS Development Following RLP Transmission by Digestive Gland Injection and Bath Exposure

No significant difference in condition index was observed between tanks at day 0 (ANOVA, $p = 0.29$) and only one individual (in one of the replicates to receive IPEH by bath treatment) showed slight body shrinkage. Mortality in the group injected in the digestive gland with IPEH began during wk 27 and reached 90% at termination (Fig. 2). Mortality began during wk 33 in the tanks to which infected black abalone were added (positive control) and cumulative mortality also reached 90%. Mortality in the IPEH bath treatment group began during week 35 and steadily increased to 70% by termination of the study. Nearly all of the animals that died in the positive control, IPEH injected and IPEH bath treated groups showed signs of WS and the presence of RLPs (see below). One animal in the control, FSW-injected group was sacrificed on day 44 post-initiation due to morbidity related to shell damage. Microscopic examination of stained tissue sections indicated an absence of RLPs in this animal. One animal in the IPEH filtrate-injected group was sacrificed on day 91 post-initiation due to the presence of an extensive shell fungal lesion (not seen in any other animals throughout the experiment). Histological examination indicated the presence of several small foci of RLP inclusions in the postesophagus. An antibiotic treatment regimen was initiated in this treatment group to eliminate any possible infections in the remaining animals. This allowed for continuity of the treatment group as a test for whether the pathogen causing WS could be a viral agent. Several wk before termination of the experiment two animals died in the negative control treatment group and histological examination revealed an absence of RLPs.

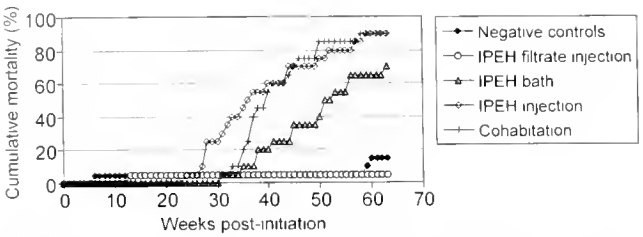

Figure 2. Cumulative mortality in Experiment 4. Treatment groups consist of duplicate tanks containing ten abalone each; the negative controls group consists of one tank of untreated abalone and one in which digestive glands were injected with filtered seawater. IPEH: RLP-infected black abalone postesophagus homogenate.

Nearly all of the animals that died had moderate to severe signs of WS. Figure 3 illustrates that mean values for body shrinkage, digestive gland metaplasia, digestive gland atrophy and foot degeneration were all higher for animals that died than those that survived. Coinciding with these elevated signs of WS, animals that died had mean RLP burdens more than four times those of survivors (RLP intensity = 2.3 ± 1.0 vs. 0.50 ± 0.8 in postesophagi, 1.8 ± 1.0 vs. 0.30 ± 0.6 in digestive glands for animals that died vs. survivors respectively, mean ± s.d.).

Histological examination of survivors of the experiment revealed that eight of the nine animals in the untreated negative control tank contained RLPs, including seven with mild infections (RLP intensity = 1 in postesophagus, digestive gland, or both) and one with a moderate infection (RLP intensity = 2 in both the postesophagus and the digestive gland). One of the tanks of IPEH filtrate-injected animals contained an individual with a light RLP infection of the postesophagus. Table 1 indicates that, despite these

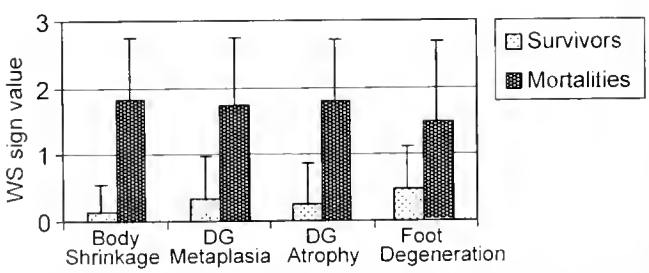

Figure 3. Severity of signs of WS in survivors and animals that died in Experiment 4. The range for each sign was from 0 (normal, healthy) to 3 (severe) (see Methods). DG: Digestive gland. Mean + s.d.

TABLE 1.

Experiment 4, mean values for RLP infection intensity and WS signs in duplicate tanks of each treatment.[1]

Treatment	Postesophagus RLP Intensity	Digestive Gland RLP Intensity	Digestive Gland Metaplasia	Digestive Gland Atrophy	Foot Degeneration	Body Shrinkage	Condition Index
Negative controls[2]	0.9, 0	0.6, 0	0.1, 0.5	0.5, 0.5	0.3, 0.9	0.3, 0.3	0.155, 0.173
IPEH filtrate injection	0, 0.2	0, 0	0.1, 0.1	0, 0.1	0.1, 0.4	0, 0.2	0.148, 0.159
IPEH bath	2.1, 1.6	1.4, 1.7	1.1, 1.8	1.3, 1.4	0.6, 1.3	1.0, 1.3	0.130, 0.131
IPEH injection	2.2, 3.0	1.5, 2.2	1.7, 2.2	1.6, 1.9	1.5, 2.2	1.9, 2.3	0.108, 0.115
Cohabitation	2.2, 2.4	1.8, 1.9	1.6, 1.6	1.7, 1.8	1.2, 1.6	1.2, 2.1	0.104, 0.122

[1] Definitions and scales are described in Materials and Methods section.
[2] First replicate = animals injected with filtered seawater, second replicate = untreated.

infections, the negative control and IPEH filtrate-injected groups had dramatically lower RLP infection intensities and signs of WS than the IPEH bath, IPEH injected and positive control groups. The mean values for RLP burden and all WS signs were highest in the cohabitation positive control group and IPEH injected group, and lower in the IPEH bath treatment group (Table 1). Table 2 shows the results of contingency table analyses comparing RLP burdens and severity of WS signs in the treatment and positive control groups to the negative controls. The IPEH injected, IPEH bath and cohabitation exposures resulted in significantly increased RLP infection intensity and severity of WS signs while the IPEH filtrate injected animals were not different from the controls.

The relationship between WS signs and RLP burdens was addressed by comparing the severity of WS signs in animals with different RLP infection intensities. Figure 4 illustrates that animals with higher RLP burdens had more severe signs of WS. This association was statistically examined by contingency table analyses which compared the frequencies of low vs. high RLP burdens in animals having low vs. high levels of WS signs. Body shrinkage, digestive gland metaplasia, digestive gland atrophy and foot degeneration all showed highly significant relationships with postesophagus and digestive gland RLP infection intensities ($p < 0.001$ for all comparisons).

The amount of food eaten on a per-weight basis by the animals in each tank was measured at five time points during the experiment. By wk 30 post-initiation, the amount of kelp eaten by the cohabitation and IPEH injection groups was lower than that of the other groups (Fig. 5). These low rates remained fairly consistent over time as severely affected animals died and others developed more severe signs of WS. Feeding rates in the IPEH bath treatment group declined prior to onset of mortality with high RLP burdens.

The renal coccidian, *Pseudoklossia haliotis* was present in only five animals with no relation to treatment across all treatment groups.

DISCUSSION

Withering syndrome appears to be directly caused by a gastrointestinal Rickettsiales-like prokaryote that has been designated "*Candidatus Xenohaliotis californiensis*" (Friedman et al. 2000). These studies demonstrate that the organism can be transmitted by experimental methods and that successful transmission appears requisite for development of WS in previously healthy red abalone.

Despite rigorous preventative efforts, we experienced apparent contamination of tanks with the RLP or the inadvertent use of infected recipient animals, as evidenced by discovery of the RLP in negative control animals in Experiments 2 and 4. In Experiment 2, the single positive animal in the FSW-administered negative control group may have resulted from infection prior to collection from the wild population; 15 months after collection of the animals in this experiment the RLP was found in a high percentage of animals at a nearby site (C. S. Friedman & C. A. Finley, unpublished observations). The two animals inoculated with IPEH that were found to be RLP-positive could have acquired infections by that treatment or via the method of the contaminated control animal. In Experiment 4, infections in the untreated negative control tank apparently developed very late in the study and were likely due to recent contamination from adjacent tanks, equipment, water

TABLE 2.

Experiment 4, significance values for Chi Square or Fisher Exact tests using dichotomous categories for each parameter (scores of 0 or 1 vs. 2 or 3) and comparing treatment groups shown to negative control groups.[1,2]

Treatment	Postesophagus RLP Intensity	Digestive Gland RLP Intensity	Survival[3]	Digestive Gland Metaplasia	Digestive Gland Atrophy	Foot Degeneration	Body Shrinkage
IPEH filtrate injection	1.0	1.0	0.605	[4]	0.231	0.487	0.487
IPEH bath	<0.001*[5]	0.005*	0.003*	<0.001*	0.251	0.052	0.117
IPEH injection	<0.001*	<0.001*	<0.001*	<0.001*	0.009*	0.003*	<0.001*
Cohabitation	<0.001*	<0.001*	<0.001*	<0.001	0.022*	0.018*	0.003*

[1] The Chi square analysis defaulted to Fisher exact test if the number of observations in any cell was less than five.
[2] Definitions and scales are described in Materials and Methods section.
[3] Survivors vs. animals that died during the experiment.
[4] All values for both treated and control groups fell into the category of low severity.
[5] Asterisks indicate significant p values ($p < 0.05$).

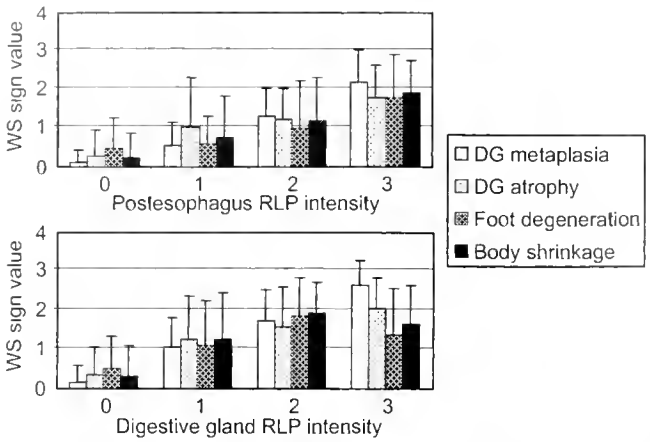

Figure 4. Severity of various WS signs for animals in Experiment 4 grouped by intensity of postesophagus or digestive gland RLP infection. The range for each sign was from zero (normal, healthy) to 3 (severe) (see Methods). DG: Digestive gland. Mean + s.d.

source or food supply. The water source (BML) and site of kelp collection (Bodega Bay) are believed to have been RLP-free throughout the study period. The two animals in one tank of the IPEH filtrate-injected treatment of Experiment 4 could have obtained infections via the routes noted above or by passage of the RLP through the filters used to create the filtrate. As commonly reported for RLPs of molluscs, "*Candidatus Xenohaliotis californiensis*" is pleomorphic with spherical to rod-shaped forms. The smallest diameter of the rod-shaped form measured 145 nm (Friedman et al. in press) and therefore the RLP may have passed through the 0.2 μm filters used on day 0.

Evidence for the RLP being the Etiologic Agent of WS

Our experiments clearly show that the agent of WS was present in RLP-infected black abalone postesophagus tissue homogenate (IPEH). Although contamination of negative control tanks prevents concluding with certainty, the dynamics of infection and mortality in the bath and injection treatments of Experiment 4 indicate that the RLP infections and WS signs were due to our experimental transmission. The onset of mortality in the IPEH-injected group before the cohabitation group suggests that this experimental treatment was more effective in transmitting the WS-associated pathogen than natural exposure. Furthermore, the lack of expression of WS in animals receiving injections of the IPEH filtrate, in contrast to those receiving unfiltered IPEH, indicate that the etiologic agent of WS is probably not a virus. These observations, considered with the relationships between RLP intensity and severity of WS signs, provide strong evidence linking the RLP directly to WS.

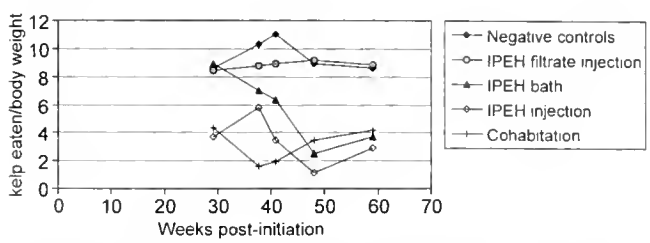

Figure 5. Relative feeding rates of Experiment 4 treatment groups measured at five timepoints. Each point is the average of values for two tanks. Abscissa scale is equal to that of Figure 1. IPEH: RLP-infected black abalone postesophagus homogenate.

WS Signs

Body shrinkage, foot muscle atrophy and to some extent digestive gland atrophy are generic signs of starvation and not specific to WS. Shrunken abalone with soft flesh have been reported in association with apparent poor food supply or environmental conditions (Young 1964, MacGinitie & MacGinitie 1966) and diseases other than WS (Nakatsugawa et al. 1999). In contrast, digestive gland metaplasia, in which functional tissue including secretory cells is replaced with cells similar in appearance to those of transport ducts, appears to be pathognomonic for WS. One fascinating aspect of this alteration is that the RLP infects duct-like tissue of the digestive gland and not terminal tubules; thus, infection with the RLP results in morphological changes which provide more tissue of the type which the pathogen can infect. Moore et al. (2000) recently reported that WS-positive animals ate less than animals without overt signs of the disease. Based on those findings we initiated feeding studies during wk 29 of Experiment 4, and the five trials suggest that a sharp drop in feeding rate precedes death in animals with signs of WS. These observations suggest that although the RLP results in pathologic changes to gastrointestinal epithelia, malabsorption resulting from these changes is not solely responsible for body shrinkage, and decrease in the ability or desire to consume food also plays a role. Diminished feeding rate may be one of the earliest indicators of adverse health in animals that acquire withering syndrome.

RLP has Low Host Species Specificity

Transmission of the RLP by cohabitation of black abalone donors and recipients was reported by Friedman et al. (this volume). Our Experiment 2 had similar results and Experiment 4 showed for the first time that the agent can be transmitted by cohabitation of black abalone donors and red abalone recipients, while Experiment 3 demonstrated transmission between red abalone. These findings agree with a wealth of morphological (Friedman et al. 1997, Moore et al. 2000), ultrastructural (Friedman et al. 2000, C. S. Friedman & J. D. Moore, unpublished observations) and DNA-based studies (Andree et al. 2000, Antonio et al. 2000) indicating that the RLP found in red and black abalone does not differ between species. RLPs identical in appearance and tissue location by histology have also been observed in California green (*H. fulgens*) and pink (*H. corrugata*) abalone (C. S. Friedman, unpublished observations).

Modes of RLP Transmission

Intramuscular injection and oral inoculation were investigated as methods of transmitting the RLP in Experiments 1 and 2. Lack of detection of the RLP following intramuscular injection agrees with observations that infected tissue appears to be completely restricted to specific cell types in the epithelium lining the digestive tract. However, Gulka and Chang (1984) reported successful transmission of a gill epithelium-specific RLP in the scallop *Placopecten magellanicus* by intramuscular injection of the adductor muscle with RLP-infected gill homogenate. The limited success of oral inoculation in our experiments may have been affected by their short duration (60 and 84 days for Experiments 1 and 2 respectively), since histological detection of the RLP was based on examination of a single 5 μm tissue slice for each potentially

affected organ. Prior to this study, transmission of the RLP had only been achieved by cohabitation. Abalone are gregarious animals, and during cohabitation frequently come in direct physical contact with each other. The results from Experiment 3 suggest that transmission of the RLP can survive in seawater and does not require close contact for transmission.

Many if not all Rickettsiales-like prokaryotes of marine invertebrates and fish are capable of direct horizontal transmission from host to host. This is in contrast to most Rickettsiales-like organisms with mammalian primary hosts, which require a parasitic arthropod host for dispersion (Marchette & Stiller 1982). The requirement for parasitic arthropods (lice, ticks, fleas) for transmission of mammalian RLPs is related to their lack of a sporogonic stage and incapability of surviving desiccation. Ingestion of RLP-contaminated food is likely the predominant mode of RLP transmission in the marine environment. Transmission of *Piscirickettsia salmonis* has been reported for coho salmon held in aquaria with infected conspecifics for one month (Cvitanich et al. 1991). Horizontal transmission of RLPs by feeding infected tissue of conspecifics has been reported for 'Stained prawn disease' of *Pandalus platyceros* (Bower et al.1996) and for transmission of an RLP from the red shrimp *Penaeus marginatus* to the blue shrimp *P. stylirostris* (Brock et al. 1986). Ingestion of contaminated food as a mode of pathogen entry and fecal/oral spread as a mode of dispersal are likely operative for the RLP associated with withering syndrome in abalone.

We found that injecting the digestive gland with infective material was an efficient mode of transmission of the RLP. Sites of injection healed rapidly with no associated morbidity or mortality. The digestive gland of abalone is a large organ containing transport ducts that are host tissue for the RLP. These open into the stomach, which receives food from the postesophagus, while the distal portions lead to terminal tubules where nutrient absorption and enzyme secretion occur (Bevelender 1988, Campbell 1965, Voltzow 1994). Among IPEH-injected animals, 16 out of 18 animals for which histological sections of both organs were available had RLPs present in both the digestive gland and postesophagus, indicating that the infections were able to spread from the former to the latter tissue. In the remaining two animals the RLP was detected in postesophagus and not digestive gland tissue, possibly due to the pathogen spreading to the postesophagus before spreading completely throughout the digestive gland. Thus, our studies provide further evidence that '*Candidatus Xenohaliotis californiensis*' is the etiologic agent of WS, a disease capable of affecting multiple haliotid species.

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SYMBIONTS OF CULTURED RED ABALONE *HALIOTIS RUFESCENS* FROM BAJA CALIFORNIA, MEXICO

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ABSTRACT Although the commercial culture of the red abalone *Haliotis rufescens* started in Baja California in 1993, no descriptions of the symbionts of this species exist. To determine which symbionts were associated with cultured red abalone in the Bahía de Todos Santos, Baja California, we conducted a survey of apparently healthy and moribund abalone. Apparently healthy abalone were larger (34 mm) than the moribund abalone (25 mm). Two polychaetes belonging to Spionidae and Serpulidae were found on the shell, although no serious damage was observed in relation to these worms. The renal coccidian *Margolisiella* (= *Pseudoklossia*) *haliotis*, was prevalent in 72% of the moribund and 10% of apparently healthy abalone. Rickettsiales-like prokaryotes (RLPs) associated with the withering syndrome (WS) were found in the epithelia of the digestive gland of all moribund abalone, 84% of apparently healthy abalone were also infected with RLP, although no clinical signs were observed. Ciliate protozoa were observed in the lumina of the esophageal pouches of both apparently healthy (78% prevalence) and moribund animals (57% prevalence). Other ciliates were observed on branchia and within the branchial cavity of apparently healthy abalone (17%) and moribund abalone (88% prevalence). Although there was a trend for greater prevalence and intensity of symbionts in moribund compared to apparently healthy abalone, the differences were not significant.

KEY WORDS: red abalone, symbionts, Rikettsiales-like prokaryotes, *Margolisiella* (*Pseudoklossia*) *haliotis*, withering syndrome, aquaculture

INTRODUCTION

In Baja California, México, abalone culture is a promising industry that has increased in magnitude over the past years (Flores et al. 1995). Successful aquaculture requires a knowledge of the symbionts of the cultured species, and which of these may present a risk for the cultured animals. More than 20 species of abalone symbionts have been recorded around the world (Table 1). The pathogenicity of symbionts varies from none to severe, and some symbionts are opportunistic pathogens (see references in Table 1). Withering syndrome (WS) is an emerging disease that affects the black abalone *Haliotis cracherodii* and red abalone *Haliotis rufescens* (Friedman 1991, Culver & Richards 1992, Friedman et al. 1993, Friedman et al. 1995, Friedman et al. 2000). The renal coccidian *Margolisiella* (= *Pseudoklossia*) *haliotis* (Dresser & Bower 1997) was first investigated as a possible causal agent of the fatal syndrome. However, no correlation was found between the WS and infection by the coccidian (Friedman et al. 1995). Lately, a prokaryote belonging to the order rickettsiales (Rickettsiales-like prokaryotes, RLP), which has been identified provisionally as "Candidatus *Xenohaliotis californiensis*", is now considered the causal agent of WS (Friedman et al. 2000). Another symbiont that has emerged as an important pest of the red abalone, *H. rufescens* cultured in California, is the introduced sabellid worm *Terebrasabella heteronuncinata* (Fitzhugh & Greg 1999), which has caused significant damage to the abalone industry (Oakes & Fields 1996, Kuris & Culver 1999). Since there have been no studies on the diseases and symbionts of abalone in México, we investigated the diseases (particularly the withering syndrome) and symbionts of apparently healthy and moribund abalone from a culture facility. In addition, we examined empty shells for external symbionts and signs of disease.

MATERIALS AND METHODS

The culture of red abalone was carried out in a protected area of the inner side of Islas de Todos Santos, one of the two islands located at the mouth of the Bahía de Ensenada (31°45′–31°59′ N, 116°36′–116°45′ W). The culture system consisted of plastic net cages suspended from floating long-lines supported by 200 L plastic barrels (Flores et al. 1995). In December 1997, 21 apparently healthy and 30 moribund abalone, and 313 empty shells were collected randomly from the bottom of a culture cage where some mortality was observed, for examination (this facility is not in operation anymore). These abalone were progeny of brood stock imported from California.

The total shell length of all abalone and empty shells was measured. In addition, the total weight (TW) including visceral, muscle, and the meat weight (MW) including visceral mass, were recorded to obtain a condition index of apparently healthy and moribund abalone. The condition index (CI) was measured as the ratio of the meat weight to total weight (MW/TW) (Friedman et al. 1997). Values obtained were grouped as suggested by Friedman et al. (1997): (1) healthy abalone with CI of $\geq$ 0.6, (2) slightly shrunken abalone with a CI from 0.55 to 0.59, and (3) severely atrophied animals with a CI < 0.55.

The inner and outer shells were checked for epibionts under a dissecting microscope. The prevalence of symbionts was estimated as the number of infested abalone/number of abalone examined ×100. Serpulid infestation was qualified, according to the percentage of shell surface covered, as low (1 to 25%), medium (26 to 50%), or high (51 to 75%). Shell blisters caused by burrowing worms (*Polydora*) were also counted.

The visceral mass of the abalone was fixed whole in Davidson fixative (Shaw & Battle 1957) for at least 24 hours. Four transverse sections containing portions of the digestive tract (posterior esophagus included), kidney, muscle and gills, were processed for paraffin histology. Deparaffinized, 5μm sections were stained with iron hematoxylin, eosin, fuchsin acid and aniline blue (Gray 1954). The degree of renal coccidian infection was estimated at 200× magnification and ranked in accordance with Friedman et al.

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TABLE 1.

Diseases and symbionts of abalone species (modified from Shields & Perkins 1997).

Abalone species	Symbiont	Interaction	Disease	Reference
Virus				
Nordotis discus	Virus?	–	Tumor-like	Harada et al. 1993
Bacteria				
Haliotis spp.	Intracellular prokaryote Similar to Rickettsiales "Candidatus *Xenohaliotis californiensis*"	–	Possibly Withering syndrome	Gardner et al. 1995, Alsatt et al. 1996, Alvarez-Tinajero et al. 1999, Friedman et al. 2000.
H. discus hannai	*Vibrio fluvialis*-II	–	Pustule disease	Taiwu et al. 1998
H. rufescens, H. kamtschatkanna, H. midae	*Vibrio alginolyticus*	–	Juvenile vibrosis	Elston and Lockwood 1983, Dixon and Hecht 1991
H. midae	*Clostridium lituseberense*	–	Clostridium infection	Dixon and Hecht 1991
Fungi				
H. sieboldii	*Haliphthoros milfordensis*	–	Mycosis	Hatai 1982
H. iris, H. australis, H. virginea virginea	Fungi	–	Fungi infection	Friedman 1997, Grindley et al. 1998
H. cracherodii	*Debaryomyces hansenii*	?	?	Gardner et al. 1995
Protozooans				
H. cracherodii	*Flabellula citata* and *Clydonella rosenfieldi*	?	?	Gardner et al. 1995
Haliotis spp.	*Margolisiella* (= *Pseudoklossia*) *haliotis*	–	Coccidiosis	Friedman et al. 1995 Dresser and Bower 1997
H. kamtschatkana, H. rufescens	*Labyrinthuloides haliotidis*	–	Infection by *L. haliotidis*	Bower 1987a, Bower 1987b
H. laevigata, H. rubra, H. cyclobates, H. scalaris	*Perkinsus olseni*	–	Perkinsiosis	Goggin and Lester 1995
Sponges				
H. cracherodii, H. rufescens, H. tuberculata	*Cliona celata californiana*	–/0	Infestation by Cliona	Hansen 1970, Clavier 1989
Flatworms				
H. discus hannai	*Proctoeces* sp.	–	Trematode parasitism	Shimazu 1970
H. tuberculata	Trematode	–	Trematode parasitism	Crofts 1929
H. fulgens	Opecoelidae	–	Red disease	Romero 1996
H. corrugata, H. fulgens, H. cracherodii	*Echinocephalus pseudouncinatus*	–	Nematode parasitism	Milleman 1951, Milleman 1963
Snails and Clams				
H. roei	*Patelloida nigrosulcata*	0		Scheibling et al. 1990
Haliotis spp.	Pyramidellid snails	–	Pyramidellid infestation	Roberts and Orr 1961
H. fulgens, H. corrugata	*Lithophaga aristata* *Lithophaga plumula*	–/0	Boring clams infestation	Alvarez-Tinajero et al. 2000
H. cracherodii, H rufescens	*Penitella conradi*	–/0	*Penitella* infestation	Hansen 1970, Alvarez-Tinajero et al. 2000
Annelid Worms				
Haliotis spp.	*Terebrasabella Heterouncinata*	–	Sabellid pest	Oakes and Fields 1996 Fitzhugh and Greg 1999
H. diversicolor aquatilis, H. kamtschatkana, H. tuberculata	*Polydora armata, P. websteri, P. flavata orientalis, Polydora* spp.	–/0	Mud worm or blister worm infestation.	Hansen 1970, Kojima and Imajima 1982, Clavier 1989, Blake 1996.
Haliotis spp.	*Broccardia knoxi*	–/0	Mud worm or blister worm infestation.	Handley and Bergquist 1977
Crustaceans				
H. asinina, H. coccoradiata, H. squamata	Pea crabs	0		Geiger and Martin 1997
H. fulgens, H. corrugata.	Copepods	0		Alvarez-Tinajero et al. 1999
Haliotis spp. from California	*Betaeus harfordi*	0		Chace and Abbott 1980

Interaction level: – negative, 0 neutral, ? unknown. –/0 negative or neutral depending on the infestation degree.

(1997): (1+) no coccidia, (2+) 1–10 coccidia per field of view, (3+) 11–100 coccidia per field, (4+) 101–1000 coccidia per field and (5+) >1000 coccidia per field. Similarly, RLP infection intensities were ranked according to the number of bacterial foci into the following categories: (1+) no RLP, (2+) 1–10 RLP, (3+) 11–100 RLP, (4+) 101–1000 RLP and (5+) > 1000 per field of view at 200× magnification. The appearance of the digestive gland was also quantified following similar criteria to Friedman et al. (1997). A score of (1+) was given if tissues were normal (Bevelander 1988), (2+) if moderate degeneration of the tissues was observed, and (3+) for severe degeneration of the tissues. Protozoa in the esophageal pouch and gill branchiae were counted from each histological preparation.

RESULTS

The mean shell length (±SE) of apparently healthy abalone was 34.4 mm (±1.03), whereas that of moribund animals was 25.9 mm (±0.67) and empty shells 24.0 mm (±0.2). An ANOVA comparing the means indicated no significant difference in shell length between moribund abalone shells and empty shells, although these groups' shells were significantly smaller than those of apparently healthy abalone (Student-Newman-Kleus, $P < 0.001$). Apparently healthy abalone were active, responded to tactile stimulation, while the color and consistency of their foot muscle were considered normal. Moribund abalone were weak, retracted and had a flaccid foot muscle. They also showed slow tactile responses.

Analysis of the shell revealed the presence of two polychaete symbionts, one belonging to the family Serpulidae (spirorbids), and the other to the Spionidae, probably from the genus *Polydora*. The spirorbids formed calcareous tubes on the external surface of the shell. Respiratory holes were not occluded and neither deformation of the shell, nor perforations toward the internal side of the shell were caused by these worms. They were present on all observed shells (in all groups), shell coverage ranging from medium to high. The prevalence of spionids in healthy abalone was 10% with an intensity of 2 worms per host; in moribund abalone, prevalence was 7% with an intensity of 1.5 (±SE 0.12), and in empty abalone shells prevalence was 7.3% with an intensity of 2.5 (±SE 32).

The histopathological study revealed the presence of macrogametes (Fig. 1a) and microgametes (Fig. 1b) of a renal coccidian parasite in the left and right kidneys. Coccidia were present in 72% of moribund and 10% of healthy abalone. The degree of infection in the moribund group ranged from moderate to heavy (4–5+) whereas it was moderate (4+) in apparently healthy abalone.

RLPs were found in the epithelial cells of the digestive tract, including the posterior esophagus, digestive diverticuli (Fig. 2a) and intestine. They were present in all moribund animals and 84% of the apparently healthy abalone. Apparently healthy abalone infected by RLP showed no detectable clinical signs of the WS. The degree of infection in moribund animals ranged from mild to heavy (3–5+), and 64% of them showed degeneration of the digestive gland ranging from moderate (2+ in 28%) to severe (3+ in 72.2%). In heavily infected animals, expelled inclusion bodies were found in the lumina of the digestive tract (Fig. 3b). In contrast, 31% of the apparently healthy infected abalone presented only mild digestive gland degeneration (2+). Apparently healthy abalone weighed significantly more than moribund abalone (6.62 g SE ± 0.54 vs. 1.84 g SE ± 0.11 respectively, *t*-test, $P < 0.001$). The condition indices of apparently healthy abalone (0.6) were higher than those recorded for moribund abalone (0.34), but were similar between apparently healthy without and with (0.6) RLP infections (Kruskal Wallis test, $H = 31.6$, $P < 0.001$ and Dunn's pairwise multiple comparison method $P < 0.005$). These condition values correlated with degeneration of the digestive gland observed in moribund animals.

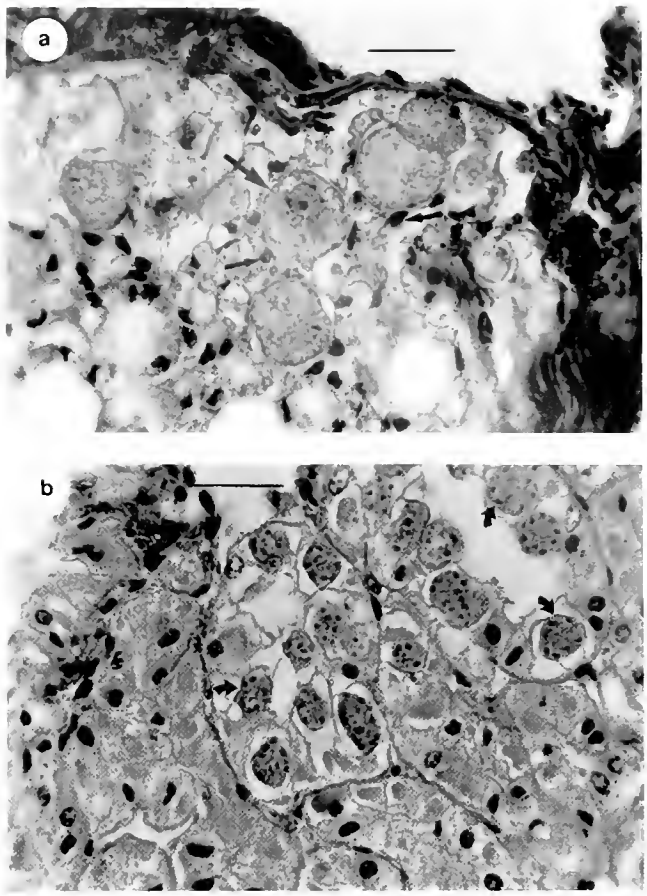

Figure 1. (a) Macrogametes of *Margolisiella* (*Pseudoklosia*) *haliotis* (large arrow) in the left kidney of a healthy abalone. Small arrows are showing hemocite around the parasite. (b) Microgametes of *M. haliotis* (arrows) in the right kidney of a moribund abalone. Scale bar = 20 μm.

A ciliate protozoan with a granular endoplasm and large vacuoles was found in the lumen of the esophageal pouches (Fig. 3a, b). The unidentified ciliate was pleomorphic and measured 40 × 12 to 90 × 19 μm. The ciliate was occasionally observed in contact with the epithelial cells of the esophagus, but was generally free in the lumen of the esophageal pouches. Prevalence in apparently healthy abalone was 78%, compared to 57% in moribund abalone. The maximum intensity of the protozoan in histological sections was 330 (mean 50.9 ± SE 18.8) in apparently healthy abalone and 120 (mean 24 ± SE 10.1) in moribund abalone (Mann-Whitney Rank sum test, $P > 0.05$).

Other ciliates of different sizes were observed in the branchial cavity and among the lamellae of the branchia (Fig. 4a). These organisms contained a granular cytoplasm with large vacuoles (Fig. 4b). Their prevalence in healthy abalone was 17% compared to 88% in moribund abalone. The maximum intensity of this pro-

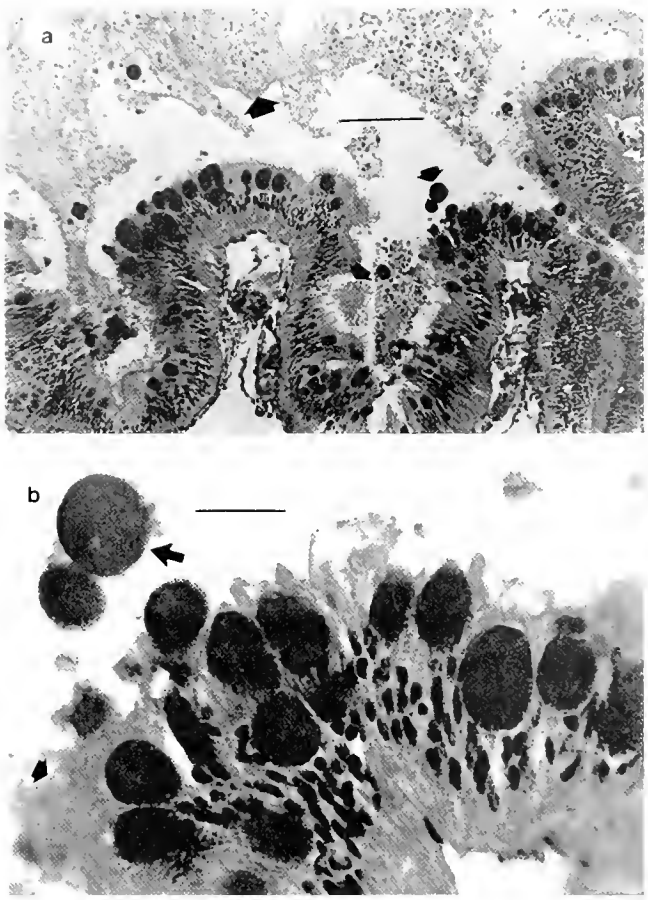

Figure 2. (a) Rikettsiales-like prokaryotes in the post-esophagus epithelial columnar cells of an infested moribund abalone. Note rupture of some columnar cells, (arrow in the middle), expelled RLP (arrow), and great quantity of mucus and hemocytes in the lumen of the post-esophagus (big arrows). Scale bar = 125 µm. (b) A close up of the infected epithelium and some expelled bacterial foci (big arrow) and rupture in the epithelium (small arrow). Scale bar = 20 µm.

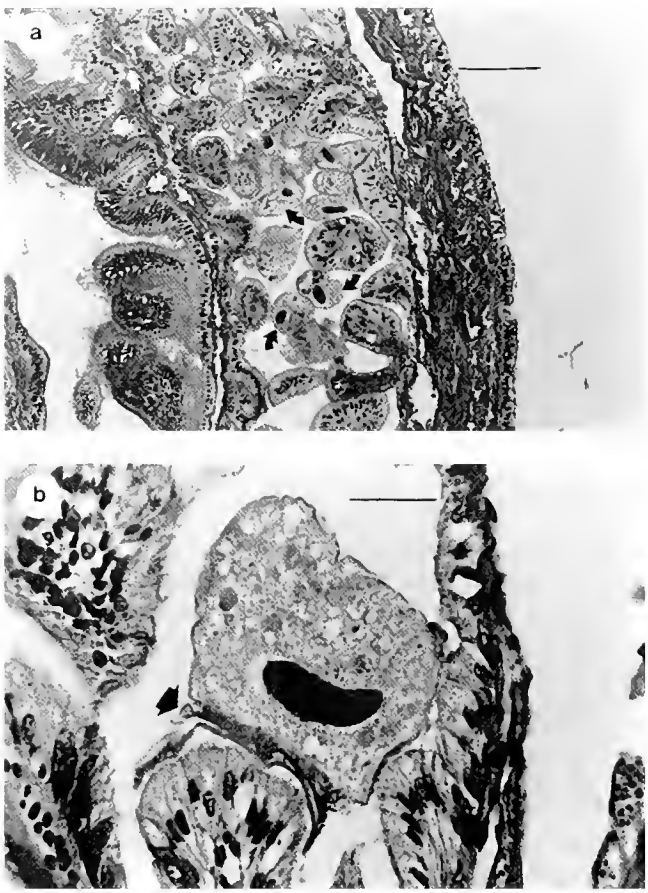

Figure 3. (a) Protozoans (arrows) in a healthy abalone's esophagous pouch lumen. Note that there is apparently no reaction of the host. Scale bar 80 µm. (b) Close up of a protozoan touching the epithelial cells of the esophagous pouch. Scale bar = 20 µm.

tozoan in healthy abalone was 21 (mean 10.2 ± SE 4.1), compared to 85 (mean 30.3 ± 6.7) in moribund abalone, although the differences were not significant (*t*-test, $P > 0.05$).

DISCUSSION

Although symbionts of wild and cultured abalone populations in California have been widely documented (Friedman 1991, Haaker et al. 1992, Lafferty & Kuris 1993, Davis 1993, Oakes & Fields 1996, Friedman et al. 1997, Shields & Perkins 1997), this is the first health evaluation of cultured red abalone from Baja California. We found the presence of a disease and several symbionts similar to those recorded in abalone from California. This is in accordance with the fact that the culture of red abalone in Baja California depends on the importation of brood and seed stock from California. Comparison of the diseases and symbionts of the cultured abalone with local red abalone populations in the wild was not possible due to the scarcity and difficult accessibility of these natural populations. These difficulties explain the need for importing red abalone from California.

There is no information on the possible effect of *Polydora* spp. in small and thin shells of young red abalone. In oysters, mussels and clams the polychaete complex, *Polydora-Broccardia*, results in loss of condition, indirect high mortality and economic losses (Lauckner 1983, Handley & Berguist 1997). Several members of the complex have been observed in *Haliotis* spp. (Hansen 1970, Blake 1996, Handley & Berguist 1997). Contrary to Clavier (1989) who reported that *H. tuberculata* smaller than 50 mm were not infested by *Polydora* spp., we found burrowing worms in individuals smaller than this size. Serpulids are commonly attached to pilings, floats, algae, mussels (Abbott & Reish 1980) and infested abalone, although they apparently do not represent any risk. There was no evidence of presence of the sabellid polychaete *Terebrasabella heterouncinata* which Fitzhugh and Greg (1999) report infests abalone in California.

Margolisiella (= *Pseudoklossia*) *haliotis* has been recorded in the black abalone *H. cracherodii*, red abalone *H. rufescens*, blue-green abalone *H. fulgens* and pinto abalone, *H. kamschatkana* (Friedman et al. 1993, Friedman et al. 1995). This coccidian has a broad geographic range that includes Bahía de Todos Santos México and the California coast. It does not seem to be lethal to these host species. This coccidian has been discounted as a causal agent of WS (Friedman et al. 1993, Friedman et al. 1995, Friedman et al. 1997). The prevalence of coccidia recorded in moribund abalone (72%) was similar to those found in abalone from central and southern California (Friedman et al. 1997). A lower prevalence and intensity of infection in apparently healthy abalone compared to moribund abalone may be coincidental. However, further

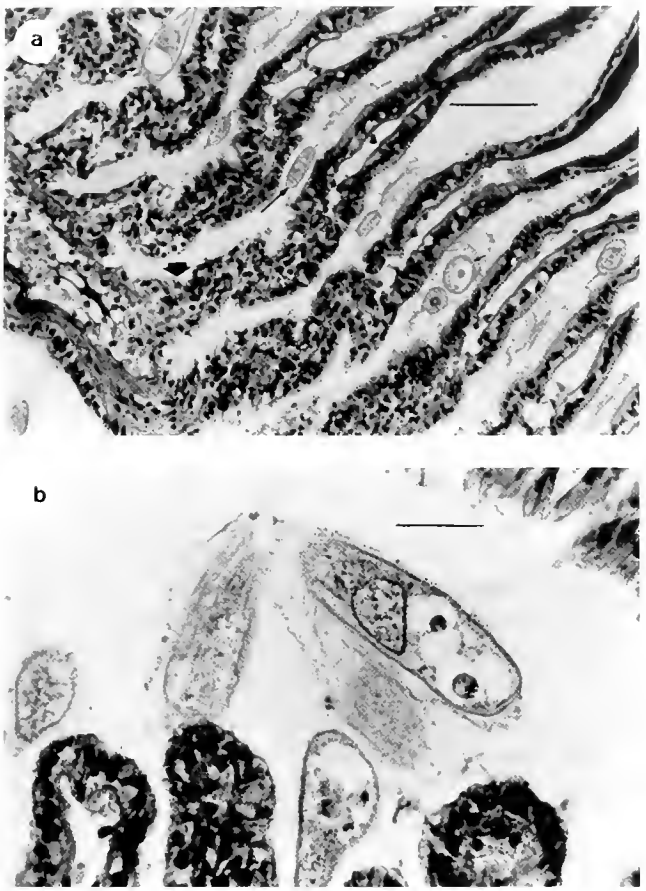

Figure 4. (a) Several ciliated protozoa (thin arrows) among the branchial filaments of a moribund abalone. Note the necrotic zones in the branchial filaments (wide arrow). Scale bar = 100 µm. (b) A close up of ciliated protozoa around the branchial filaments of a moribund abalone. Note the large nucleus and vacuoles in the protozoan. Scale bar = 20 µm.

studies on alterations in abalone physiology in relation with renal coccidia are needed.

The presence of RLP in cultured Mexican stocks of red abalone must be considered in the aquaculture facilities management and sanitary practices. The majority of the observations of WS in the red abalone were reported from laboratory/aquaculture facilities in California (Moore et al. 2000). Friedman (1995) reported that during a survey of abalone for WS in California, from 1 to 4% of the red abalone from San Miguel Island had visual signs of WS. Moribund abalone in our studies were characterized by retracted visceral tissues, discoloration and shrunken appearance of the foot muscle, low condition, presence of RLP in the digestive gland and some degeneration of the digestive gland, suggesting that these abalone were affected by WS. A similar situation has been reported by Moore et al. (1999) who associated dramatic mortality of red abalone from aquaculture facilities with the WS. Withering syndrome in black and red abalone has a long incubation period (5–7 months and 7–8 months respectively), before clinical signs are visible (Friedman et al. 1997, Moore & Friedman unpublished results cited by Friedman et al. 2000). Hence, RLP infected individuals are undetectable by visual condition. The degree of infection in asymptomatic but infected abalone was similar to those found in moribund abalone and could be explained by susceptibility differences among individuals and incubation period. Our sampling was carried out during the 1997 El Niño when temperature was 2°C above normal in the area. High water temperatures have corresponded with increased mortality of RLP-infected black abalone (Friedman et al. 1997). Undoubtedly, more thorough and detailed studies (using large samples) concerning RLP infection in different abalone species, and studies focusing on the identification and differentiation of RLP species or strains associated with abalone species are needed.

The similar prevalence and total numbers of ciliates from the esophageal pouches in healthy and moribund abalone found in the present study suggest a commensal symbiosis. Further taxonomic and biological studies are needed to determine the species of this protozoan and the nature of the symbiosis.

Vanblaricom et al. (1993) recorded the presence of suctorian ciliates in gill squashes and mantle scrapings of healthy black abalone and those apparently afflicted by the WS. Presence of these ciliates did not relate to clinical signs of the disease in the host abalone. A considerable number of ciliates has been associated with marine bivalves and the majority of these appear to be harmless commensals (Lauckner 1983). In this study, similar prevalence of ciliates in apparently healthy and moribund abalone suggested that these organisms are not related to the moribund condition of some of the abalone examined. An increase in total numbers of protozoans on the gills coincides with host weakness and death (Lauckner 1983) which may be due to the saprozoic habits of some species (Kudo 1982).

Abalone from Baja California have almost the same symbionts species as those in California. This highlights that it is necessary to maintain a continuous monitoring program to avoid introduction of new potentially dangerous symbionts to the region.

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LIFE HISTORY OF AN EXOTIC SABELLID POLYCHAETE, *TEREBRASABELLA HETEROUNCINATA*: FERTILIZATION STRATEGY AND INFLUENCE OF TEMPERATURE ON REPRODUCTION[a]

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ABSTRACT The California abalone aquaculture industry has been struggling to rid itself of an exotic sabellid, *Terebrasabella heterouncinata* following its inadvertent introduction from South Africa in the late 1980s. The development of an effective management strategy is dependent upon understanding the life history of this sabellid, including its fertilization strategy (e.g. self-fertilization) and its generation time. In the present study, red abalone, *Haliotis rufescens* Swainson 1822, with single sabellid infestations were isolated in containers at 18°C (single host and sabellid per container). This first, parental generation (P), was held in isolation until individuals produced F_1 larvae. The F_1 larvae were subsequently isolated until individuals produced a second, F_2, generation. In a separate study, uninfested red abalone were exposed to sabellid infested abalone at 11.2°C, 15.6°C, and 20.9°C, temperatures typically encountered in California. The larvae were subsequently observed as they developed to specific life stages: the initiation of feeding, the development of all 11 setigers (which closely relates to sexual maturation) and the completion of their life cycle as recognized by the production of motile, infesting, larvae. Approximately 50% of the sabellids examined at 11.2°C, 15.6°C, and 20.9°C had developed the ability to feed by day 6, 5 and 4 ($P < 0.001$), had developed all 11 setigers by day 83, 68 and 48 ($P < 0.001$) and had produced larvae by day 298, 165 and 111 ($P < 0.001$), respectively. This research demonstrates that isolated individuals do pose the threat of producing fully functional offspring and that the generation time of *T. heterouncinata* is significantly temperature dependent.

KEY WORDS: sabellid, abalone, life history, self-fertilization, *Terebrasabella*

INTRODUCTION

The California abalone aquaculture industry is presently struggling with two serious diseases, one of which is an introduced sabellid polychaete, recently described as *Terebrasabella heterouncinata* (Fitzhugh & Rouse 1999). This sabellid was cited by California growers as their most serious "problem and constraint," reporting that the reduced growth rates and negative public perception associated with the worm were having a substantial negative impact on the industry (Mcbride 1998). *Terebrasabella heterouncinata* is believed to have been introduced from South Africa in the late 1980s, via the importation of infested research abalone (Kuris & Culver 1999, Ruck & Cook 1998). Concern exists regarding the threat that the sabellid poses to the California cultured abalone industry and to native gastropod populations if it were to become established within the state's intertidal ecosystem (Kuris & Culver 1999). Presently, there is one documented case of *T. heterouncinata* infestations in gastropods (snails and limpets) adjacent to a culture facility (Kuris & Culver 1999). Broad-scale removal of infested hosts, combined with clean-up efforts at the culture facility may have curtailed the sabellid from becoming permanently established in the intertidal environment (Kuris & Culver 1999).

Infestations occur by the unique ability of *T. heterouncinata* to overcome the host abalone's defenses and settle upon the under side of the leading edge of the shell. Normal shell deposition is disrupted as the host attempts to cover the irritant with nacre. In heavily infested abalone, the deposition of the prismatic shell layer all but ceases, resulting in the domed shell found in association with the reduced growth rates (Culver et al. 1997, Kuris & Culver 1999, Ruck & Cook 1998).

Attempts to control *T. heterouncinata* in commercial settings have included manipulations of water temperatures, the coating of abalone shells with wax, quarantine of infested stocks, and improved sanitation practices (Oakes & Fields 1996, Oakes et al. 1995, Leighton 1998). Many of these techniques have been met with limited success, allowing low-level infestations to persist. Suggested new treatments include novel therapeutic delivery systems using micro-encapsulation (Ruck & Cook 1998, Shields et al. 1998) and the use of ultrasound (Loubser & Dormehl 2000). Unfortunately, none of these techniques, to date, have been successfully applied in a culture setting.

The initial description of *T. heterouncinata* noted that individuals were simultaneous hermaphrodites, but did not indicate if they were functional hermaphrodites, capable of self-fertilization. Upon settling at the 7th setiger stage, the sabellids are covered with nacre by the host resulting in the passive formation of what becomes the adult's tube. Development is characterized by the formation of a branchial crown and four additional setigers. As the 11th setiger is formed, an alteration of the type of uncini on the 6th setiger also occurs, both events occur as individuals are recognized as sexually mature (Fitzhugh & Rouse 1999). Spermatogenesis occurs in the 8th setiger and oogenesis occurs in both the 9th and 10th setigers. The mature adult broods several embryos within its tube for an unknown, temperature dependent, amount of time. The embryos eventually mature and develop into motile larvae that subsequently emerge from the tube of the adult and seek a suitable area of attachment (Fitzhugh and Rouse, 1999).

In the following study we examined the fertilization strategies and the life history of *T. heterouncinata*. We investigated if an isolated sabellid is capable of reproduction through two generations. We assessed the reproductive capabilities of *T. heterouncinata* by quantifying generation times at three water temperatures that reflect the range of temperatures encountered in California.

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MATERIALS AND METHODS

Animals and Husbandry

Commercially reared red abalone, *Haliotis rufescens* Swainson 1822, that measured 15–25 mm in shell length, were purchased from or donated by culturists in California. All animals were held in the Pathogen Quarantine Facility at the Bodega Marine Laboratory, Bodega Bay, California. All effluent produced at this facility is chlorinated (>10 ppm Cl^- for 2–3 h) and dechlorinated prior to release. Animals were reared in flow-through, full strength, sand-filtered seawater. Experiments were conducted at average mean temperatures of ambient (12.6°C), heated (15.6°C, ~18°C and 20.9°C) or chilled (11.2°C) seawater. All abalone were fed *Macrocystis pyrifera* Agardh 1820, *ad libitum* and remained under artificial light on a 12-hour dark, 12-hour light cycle for the duration of these experiments. Strict sanitation protocols were followed to insure that no cross-contamination occurred.

Single Infestations Trial 1

Fifty uninfested red abalone were placed into a 10-l container with five red abalone, heavily infested with *T. heterouncinata*, for 24 hours. The newly infested abalone were inspected with a dissecting microscope 48 hours post-exposure and infestations were quantified. Fourteen abalone with single sabellid infestations were removed and individually isolated in 200-ml containers. Four abalone, that had multiple sabellid infestations per abalone, were isolated in a similar manner to serve as positive controls. Four uninfested abalone were isolated in a similar manner to serve as negative controls. All 22 containers received ambient seawater, ~12.6°C, and were randomly placed on a wet table.

Abalone were sampled every 14 days by gently pushing aside the epipodium and mantle tissue and inspecting the leading edge of the shell for the presence of recently settled larvae. Following the discovery of new larvae, treatments were sampled every seven days. Uninfested abalone were added to any container in which the original infested host abalone had died, providing live hosts for larval settlement. The abalone were observed for a total of 32 weeks, at which point the sabellids that had not produced larvae were carefully dissected from their tubes and observed under dissecting and compound microscopes to determine if signs of reproduction were visible.

Single Infestations Trial 2

Forty uninfested red abalone were exposed to *T. heterouncinata* infested abalone as described in Trial 1. Abalone were inspected 48 hour post-exposure and the infestations were quantified. Four abalone with single parental (P) generation infestations (Fig. 1) and four uninfested abalone (negative control) were removed and individually isolated in 700-ml containers. Animals were visually inspected weekly. Sabellid larvae settle preferentially on live hosts (C. A. Finley & C. S. Friedman, unpublished observation). To allow F_1 larvae to settle only on recipient, sabellid-free abalone, infested (P host) abalone were sacrificed after 60 days post-exposure and a single uninfested abalone (larval recipient) was placed into each tank with the infested shucked shells. Once a recipient abalone became infested with an F_1 larva, the newly infested animal was removed and isolated (single abalone with single sabellid) in a 700-ml container. Additional uninfested abalone were added to the containers with the P generation sabellids to attain multiple individuals with single F_1 infestations (Fig. 1).

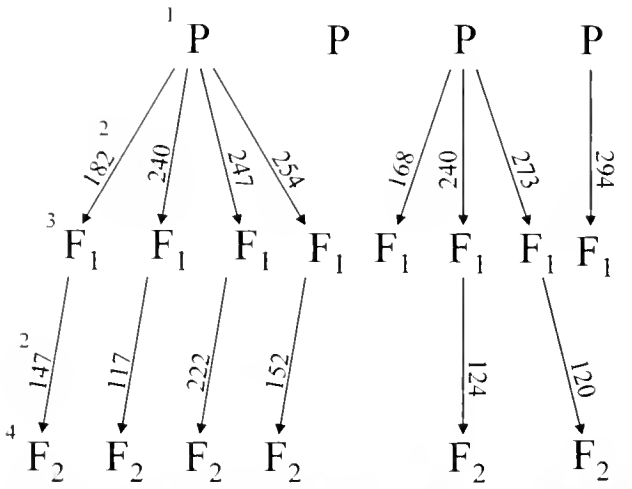

Figure 1. Individual abalone from single infestation trial 2. Two successive generations of sabellids and the number of days until offspring were produced is illustrated. Superscripts (1–4) indicate (1) the parental generation, (2) the number of days until larvae were observed, (3) the F_1 larvae produced by the parental by the parental generation and (4) the F_2 larvae produced by the F_1 generation.

Host abalone containing F_1 larvae were visually inspected for signs of F_2 larvae. All containers received heated seawater (~18°C) for the duration of this trial. The abalone were observed for a total 497 days, at which point any sabellids that had not produced larvae were carefully removed and observed for signs of reproduction.

Life History Trial

Terebrasabella heterouncinata infestations were achieved by commingling 200 uninfested and 20 infested (10 live and 10 shucked) red abalone in a 10-l container for a 24 hour period. Following the exposure the 200 newly infested abalone were divided into three replicates tanks. Exposures were conducted at 11.2°C, 15.6°C, and 20.9°C. Infestations were quantified 48 hours after the end of the exposure period. The removal of the 20 heavily infested donor abalone marked time zero and every subsequent 24-hour period was regarded as an additional day of development.

Development to the following three life stages was recorded: the ability to feed, the development of the full complement of setigers and the production of infestive, motile larvae. Replicates were sampled every 24 hours for the first eight days, followed by weekly sampling. At each sampling point, six sabellids were removed from each replicate by sacrificing the host abalone and gently breaking up the shell with a scalpel.

The ability to feed was determined by providing the sabellids with suspended stained lipid beads (LBs). Following the protocol of Shields et al. (1998) microencapsulated, sudan black-stained, tripalmatin lipid beads that ranged in size from 3 to 30 μm were produced. Several abalone, with a total of six sabellids, were removed from each replicate tank and placed into three 200-ml containers to which 0.3 g of the lipid beads were added. The seawater-lipid bead suspension was stirred every 10 minutes. After 30 minutes, the abalone hosts were sacrificed and the sabellids were excised and examined for the presence of LBs in the digestive tract using a light microscope. We enumerated the number of feeding sabellids, terminating the LB exposures when all sabellids in a given replicate were able to feed.

To identify the development of all 11 setigers, six sabellids

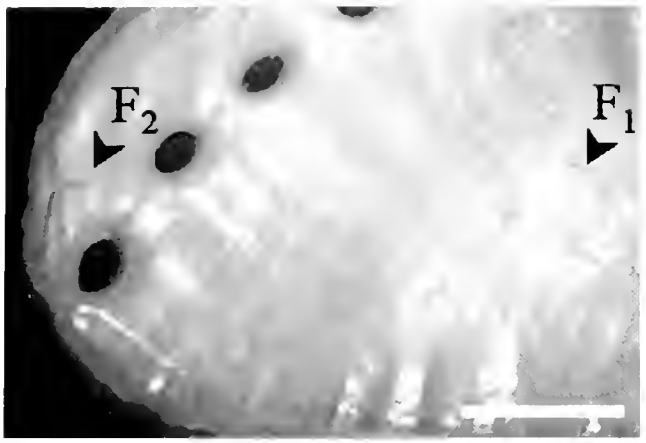

Figure 2. Abalone shell showing both the F_1 and F_2 generation. Bar = 5mm.

were removed weekly from each tank and the number of setigers was enumerated using a light microscope at 600× magnification. When 100% of the sabellids sampled from each replicate had developed a full complement of setigers, sampling was reduced to once every 1–4 weeks depending on the number of sabellids remaining and the temperature under investigation.

The completion of the life history of *T. heterouncinata* was defined by the production of either a motile, infesting larva or by a recently settled larva. Sabellids were excised as above and the presence or absence of motile or newly settled larvae was recorded. The experiment terminated when 100% of the sabellids inspected from each replicate completed their life cycle.

Statistical Analysis

With the assumptions of an ANCOVA not being met, an alternative way to analyze the data was needed. Linear regression lines were calculated for each replicate. The number of days that it took 50% of the sabellids to reach a developmental stage (T_{50}), was then calculated from the regression line using the formula $Y = bX + a$, setting the dependent variable, Y, equal to 50% and solving for X. The T_{50} estimates were then compared using one-way analysis of variance (ANOVA).

The data from the replicates at each temperature were also pooled and the number of days that it took for 50% of the sabellids to reach a developmental stage was calculated. The T_{50} estimates from the pooled replicates at a given temperature were then presented as the best estimate for the number of days post-settlement required for 50% of the sabellids to reach a given developmental stage at each temperature.

RESULTS

Single Infestations Trial 1.

Larval production by six of the 14 individually isolated sabellids began during weeks 26 to 32 (Table 1). Of the seven sabellids that had not produced any larvae, three contained well-developed internal eggs, one contained both internal and external eggs and embryos, and the remaining three tubes were devoid of sabellids at the termination of Trial 1. One abalone was contaminated by the effluent from another tank and was discarded.

Abalone with multiple sabellid infestations (positive control) also began producing larvae during week 26. Sabellids on 3 of the 4 abalone produced larvae (Table 1) and only vacant sabellid tubes were found on the fourth animal at the termination of Trial 1. No sabellid infestations were found on the negative control animals (Table 1).

Single Infestations Trial 2.

Three of the four P generation sabellids produced an F_1 generation. The first larva was observed on day 168 (Fig. 1). Subsequently, two of the P generation sabellids produced additional F_1 larvae (three and four, respectively). Six of the F_1 generation produced an F_2 generation. The first larva appeared 117 days after the F_1 parent had settled (Fig. 1). Figure 2 illustrates a host shell with both F_1 and F_2 sabellids.

Life History

Recently settled larvae that had fed on stained lipid beads were easily distinguished from individuals that had not fed (Fig. 3). The amount of time required for the sabellids to develop the ability to feed increased significantly as the temperature decreased (ANOVA, $p < 0.001$). The pooled replicates indicated that 50% of the sabellids reached this developmental stage at 20.9°C, 15.6°C, and 11.2°C by 3.7, 4.9 and 6.0 days post exposure, respectively (Fig. 4).

The rate at which the sabellids developed a full complement of setigers was also affected by temperature. The number of days required for the sabellids to develop all 11 setigers required significantly more time as the temperature decreased (ANOVA, $p < 0.001$). Analysis of the pooled data indicated that 50% of the newly settled larvae developed a complete complement of setigers at 20.9°C, 15.6°C, and 11.2°C by 47.7, 68.4 and 83.1 days post exposure, respectively (Fig. 5). The majority of the sabellids observed had developed both eggs and sperm in conjunction with the development of all 11 setigers.

The number of days required for the sabellids to produce motile, infesting larvae or newly settled larvae required significantly more time as the temperature decreased (ANOVA, $p < 0.001$). Analysis of the pooled data indicated that 50% of the newly settled larvae had completed their life history at 20.9°C, 15.6°C, and 11.2°C by 110.8, 165.0 and 297.8 days post exposure, respectively (Fig. 6).

DISCUSSION

Self-fertilization is not considered the predominant mode of fertilization for many simultaneous hermaphrodites due to the del-

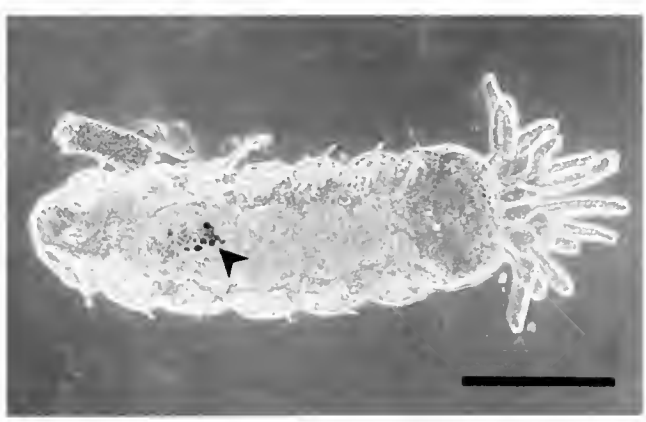

Figure 3. A juvenile sabellid with lipid-stained beads visible in the digestive tract (arrow). Scale bar = 100 µm.

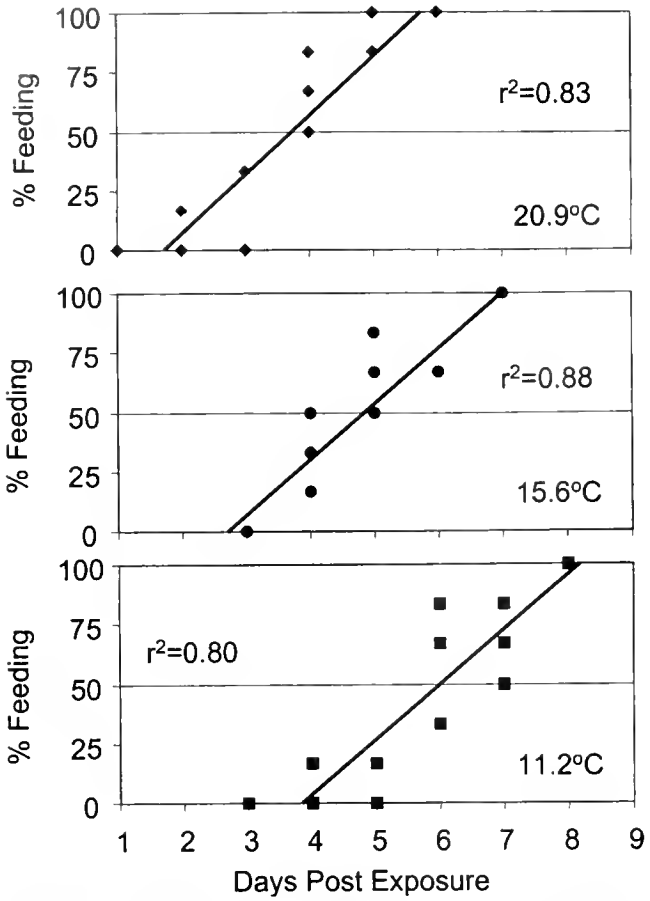

Figure 4. Percentage of feeding sabellids in pooled replicates at each of three temperatures investigated. Regression lines indicate that 3.7, 4.9 and 6.0 days post-exposure, 50% of the sabellids had developed the ability to feed at 20.9°C, 15.6°C, and 11.2°C, respectively.

eterious effects believed to be associated with inbreeding (Heath 1977, Beckwitt 1982, Michiels & Streng 1998). There are, however, several examples of successful self-fertilization among marine invertebrates (Ghiselin 1974, Beckwitt 1982, Knowlton & Jackson 1993, Scharer & Wedekind 1999), some of which include members of the class Polychaeta (Ghiselin 1969, Hsieh 1997). Hsieh (1997), for example, found no deleterious effects of self-fertilization in the simultaneous hermaphrodite *Laonome albicingillum*, also a sabellid polychaete.

Most polychaetes broadcast spawn small ova that are capable of being distributed great distances from the parent. In contrast, *T. heterouncinata*, produces a relatively small number of large eggs that are brooded within their tube and lack a pelagic stage. Combined, these characteristics make *T. heterouncinata* a likely candidate for self-fertilization (Knowlton & Jackson 1993). In addition, adult sabellids are sessile and rely solely upon their short infesting motile stage for the distribution of progeny to other hosts. Given this life history, one would predict that if the benefits of reproductive assurance associated with self-fertilization outweighed the deterrents of "selfing," the requirement of cross-fertilization would be selected against (Jarne & Charlesworth 1993). For a sessile parasite such as *T. heterouncinata*, that invests a relatively large amount of energy into a limited number of eggs combined with a lack of control over accessing a partner, self-fertilization would be a successful strategy to assure that a founding sabellid on a host abalone would remain capable of producing progeny.

Self-fertilization is one plausible explanation for the production of the F_1 and F_2 generations that we observed, though alternative fertilization strategies, such as parthenogenesis, cannot be ruled out. These experiments were designed to investigate whether a single isolated sabellid has the ability to produce functional progeny or if a minimum population number is necessary to assure cross-fertilization. Our research indicates that a single sabellid is capable of founding a viable population.

Following its accidental introduction in the late 1980's, *T. heterouncinata* was able to quickly spread throughout the state's farmed abalone industry. This rapid spread was due, in part, to the interdependency within the industry on seed transfers, in conjunction with the early misidentification of the sabellid as a native polychaete. Following the discovery of the sabellid as an exotic species, the California Department of Fish and Game (CDF&G) recognized the organism as a threat to the state's natural resources and aquaculture industry. In an attempt to control the further spread of the polychaete, the CDF&G, consulting with the industry-initiated Abalone Sabellid Worm Advisory Committee, developed a policy that all abalone transfers by farms be inspected and deemed to be sabellid-free (Aquaculture Disease Committee, July 6, 1995; Thoesen 1994). This sampling regime was selected, in part, because a minimum population density of sabellids was believed to be required to establish a viable population. Our research demonstrates that low-level infestations (or even a single founding

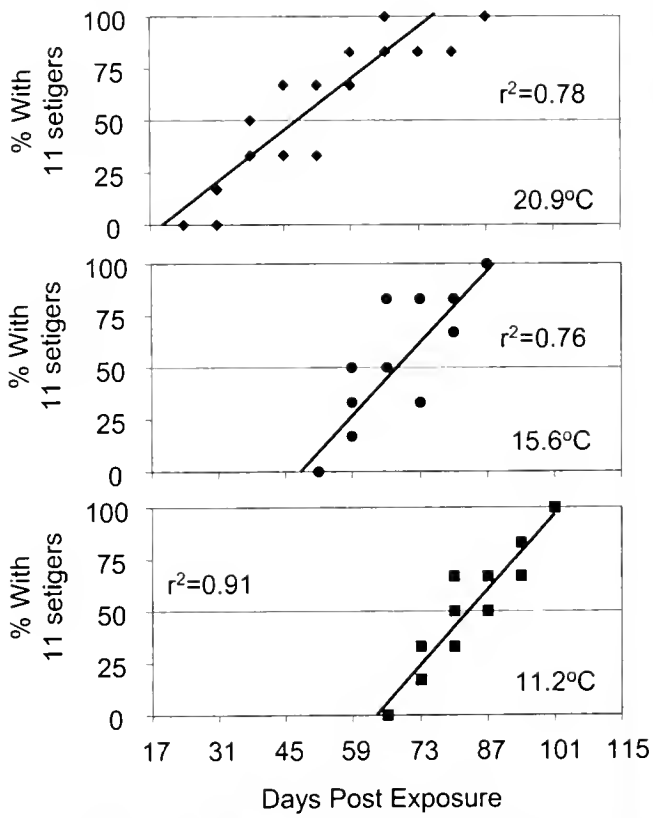

Figure 5. Percentage of sabellids that had developed a full complement of setigers (n = 11) in pooled replicates at each temperatures investigated. Regression lines indicate that 47.7, 68.4 and 83.1 days post-exposure, 50% of the sabellids had developed 11 setigers at 20.9°C, 15.6°C, and 11.2°C, respectively.

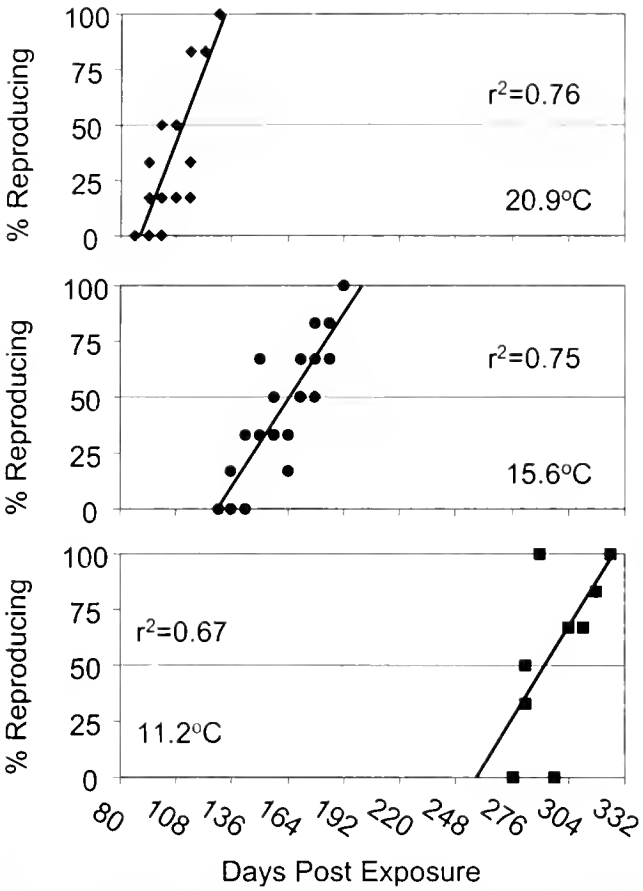

Figure 6. Percentage of sabellids that had produced motile, infestive larvae or newly settled larvae in pooled replicates at each temperature investigated. Regression lines indicate that after 110.8, 165.0 and 297.8 days, 50% of the sabellids had completed their life history at 20.9 C, 15.6°C, and 11.2°C, respectively.

sabellid) could pose a significant threat not only to the aquaculturist, but to the marine intertidal ecosystem.

Many polychaetes demonstrate positive relationships between environmental temperature, within the physiological ranges, and the rate at which they reach maturity (Cha et al. 1997, Olive et al. 1997, Qiu & Qian 1997, Qiu & Qian 1998). *Terebrasabella heterouncinata* appears to follow this trend. Previous research has reported that reproductive maturity in *T. heterouncinata* can occur in approximately one month (Kuris & Culver, 1999) or in three months (Ruck & Cook 1998) when animals were held at undefined ambient temperatures. Our controlled laboratory studies found that the most rapid development occurred at the highest of the three temperatures investigated (20.9 C), where average development of a full complement of setigers (n = 11) took 47.7 days. This decrease in time to maturity in association with elevated seawater temperatures would be expected to result in increased infestation levels, an observation that agrees with what has been experienced by California growers (B. Beede pers. comm.). Higher infestation levels are typically observed in the warmer waters of the southern part of the state, particularly in association with El Niño events.

Previous studies on polychaetes have demonstrated that at lower temperatures (again within physiological ranges) development would be prolonged, but that one would expect to observe a significant number of embryos and larvae developing into mature adults (Qiu & Qian 1997). Our research indicates that the development of all 11 setigers and the age at maturity is indeed prolonged at lower temperatures and would be expected to result in lower infestation levels. *Terebrasabella heterouncinata* does, however, appear to be capable of completing its life history at the lower temperatures encountered in the northern part of the state where annual averages range from 8–13°C (Mcbride 1998). This portion of the state should not be viewed as a thermal refuge from *T. heterouncinata*, although its life history will take longer to complete and less obvious, low level infestations, may result.

ACKNOWLEDGMENTS

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SHELL BORING CLAMS IN THE BLUE ABALONE *HALIOTIS FULGENS* AND THE YELLOW ABALONE *HALIOTIS CORRUGATA* FROM BAJA CALIFORNIA, MÉXICO

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ABSTRACT The blue abalone *Haliotis fulgens* and yellow abalone *Haliotis corrugata* support the major portion of the abalone fishery in Baja California, México. Abalone shells are marketable according to the quality of their shell, which is determined by the quantity and kind of epibionts, such as boring clams. In order to determine the extent of infestation by shell boring clams in these abalone species, shell samples from the commercial catch were obtained from Isla de Cedros, Baja California, and analyzed. Three shell boring species, *Lithophaga aristata*, *Lithophaga plumula* and *Pentella conradi*, were found infesting the blue abalone. The yellow abalone was infested by *L. aristata* and *L. plumula*. The prevalence and density of these two species were greater in the yellow abalone than in the blue abalone. The density of boring clams increased on older and consequently larger abalone shells (greater shell surface), although this trend needs confirmation. X-ray images revealed the severity of shell damage in heavily infested animals. The identification of different fishing areas that provide different quality abalone shell, due to their boring clams load, opens the possibility for determining management regulations for obtaining better shells for marketing.

KEY WORDS: boring clams, *Lithophaga* sp., *Pentella* sp., *Haliotis fulgens*, *Haliotis corrugata*, abalone

INTRODUCTION

The abalone fishery is one of the most important economic activities in Baja California, México. This is because of the great value of the foot muscle of the abalone as a delicacy for human consumption, and because of the beauty of its shell which is used in jewelry. Two species, the blue abalone *Haliotis fulgens* (Philipi 1845) and yellow abalone *Haliotis corrugata* (Gray 1828), support this fishery.

Abalone shells from Baja California are marketable according to their quality, which is closely related to epibionts load and shell appearance. Top quality shells are those that have less than 50% of shell surface covered by epibionts, and no dark spots and blisters on the inner surface. These shells are exported for jewelry to Taiwan, Korea and China, reaching a value of about US$1,500 per ton. Shells that have 50% to 80% of their external surface covered with epibionts, and blisters on their inner surface are worth around US$1,000 per ton. Shells that have >80% of their external and internal surface affected by epibionts are not exported, but are marketable locally at a low scale, reaching a value of US$750 per ton.

The abalone shell is an adequate substrate for a great variety and quantity of epibionts to settle on. Of these, the boring clams may produce severe damage, as their burrows may penetrate the inner shell of the abalone, causing the host to seal the hole internally with a leathery layer of conchiolin. Moreover, the abalone may secrete more nacreous material inside the bore site, forming a blister (Abbott & Haderlie 1980). Occasionally, a great blister may appear to be an irregular half-pearl, however, this is empty and of no marketable value. On the other hand, abalone may produce true pearls between the visceral mass and the inner shell as a reaction against foreign particles. These abalone pearls are sold locally as valuable and expensive souvenirs, but are not exported because they are rare.

There have been no studies investigating the possible association of boring clams with the abalone, even though these epibionts damage the abalone shell and thus negatively affect their marketability. In order to determine the identity and degree of infestation by shell boring clams in the blue and yellow abalone at Isla de Cedros, samples were obtained from a commercial catch. Infestation rate as a function of abalone age and shell surface area was examined.

MATERIALS AND METHODS

In November 1997, 38 blue abalone and 48 yellow abalone were collected commercially from the northern end of Isla de

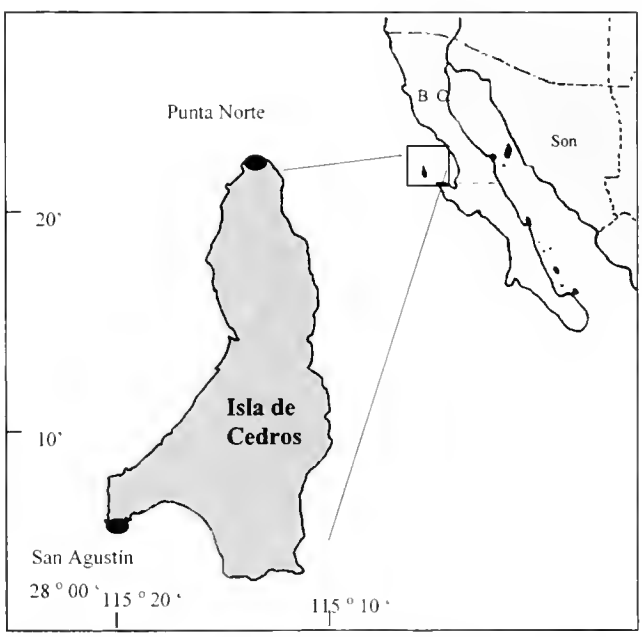

Figure 1. Sampling sites on the Isla de Cedros, Baja California, México.

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Figure 2. Abalone Shell of *Haliotis corrugata* (A) and *Haliotis fulgens* (B).

Cedros (Punta Norte). A further 13 blue abalone and 21 yellow abalone were obtained from the commercial catch at San Agustin near the southern end of the island in June 1998 (Fig. 1). Abalone foot musele and viscera were detached from the shell, and the shell was numbered, cleaned and dried. The shells (Fig. 2) were examined under a stereoscopic microscope for the presence of boring clams, including blisters and spots of conchiolin deposition. All clams were extracted from the shell with the aid of dissection needles. Larger clams were measured with an electronic caliper; and the smallest, with a micrometer placed on the stage of a stereoscopic microscope. All clams were stored in dried vials and identified (Soot-Ryen 1955, Keen & Coan 1974, Haderlie & Abbott 1980).

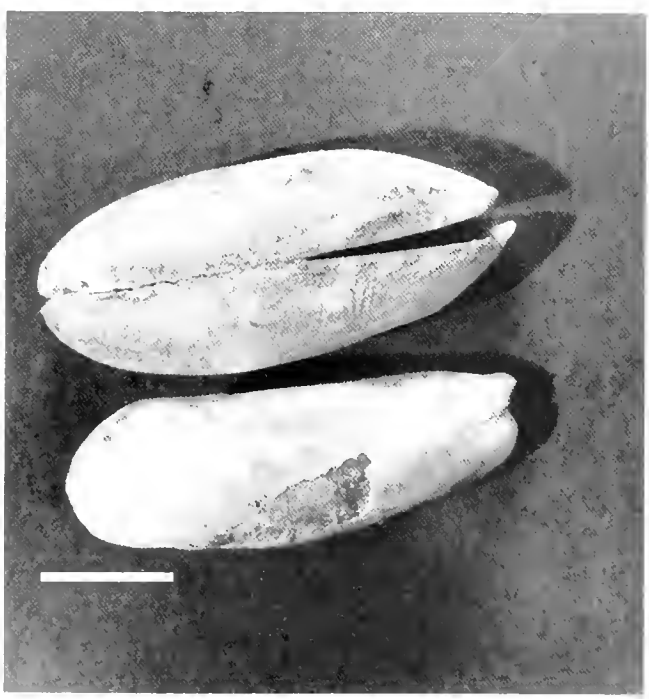

Figure 3. *Lithophaga aristata*, easily recognized by the crossing projections of the sculpture on the posterior part of the valves.

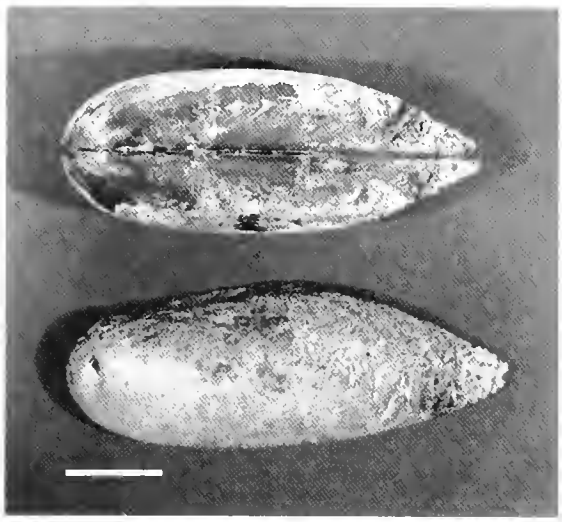

Figure 4. *Lithophaga plumula*, the most conspicuous character is the plume-like sculpture on the upper and posterior part of the valves.

The clams were grouped by species and size class to describe the composition of the epibiont clam population in the shell of each abalone species. Prevalence was estimated as the number of infested abalone/number of abalone examined ×100. Density (intensity) was recorded as the number of clams per cm^2 of abalone shell surface. The surface area of each cleaned abalone shell was estimated in the following way: aluminum foil was made to conform to the shape of abalone shells and all their irregularities. The foil was carefully cut, dried and weighed. Pieces of aluminum foil were weighed, obtaining the following least squares regression equation:

$$x = y - 0.0019/0.046$$

where

y = foil weight (g) and

x = area (cm^2); ($r^2 = 1$)

Figure 5. *Penitella conradi*, oval shaped, the shell gaping widely anteriorly.

TABLE 1.

Mean, minimum, maximum density and prevalence of shell boring clams in abalones collected in Punta Norte, Isla de Cedros B. C.

	Haliotis corrugata				*Haliotis fulgens*			
	Mean	**Sd**	**Min/Max**	**Prev.**	**Mean**	**Sd**	**Min/Max**	**Prev.**
L. aristata	0.116	0.217	0–1.057	43.7	0.077	0.351	0–2.121	13.1
L. plumula	0.060	0.152	0–0.663	25.0	0.017	0.073	0–0.396	5.3
P. conradi	0	0	0	0	0.005	0.023	0–0.138	5.3

Density.-No. Clams/cm^2 of abalone shell.
Standard deviation (Sd).
Prevalence (Prev.) % of abalone infested with boring clams.

TABLE 2.

Mean, minimum, maximum density and prevalence of shell boring clams in abalones collected in San Agustín, Isla de Cedros B. C.

	Haliotis corrugata				*Haliotis fulgens*			
	Mean	**Sd**	**Min/Max**	**Prev.**	**Mean**	**Sd**	**Min/Max**	**Prev.**
L. aristata	0.400	0.602	0–2.198	57.1	0.048	0.099	0–0.288	23.1
L. plumula	0.180	0.260	0–0.800	42.8	0.062	0.226	0–0.818	7.7
P. conradi	0	0	0	0	0.017	0.027	0–0.066	7.7

Density.-No. Clams/cm^2 of abalone shell.
Standard deviation (Sd).
Prevalence (Prev.) % of abalone infested with boring clams.

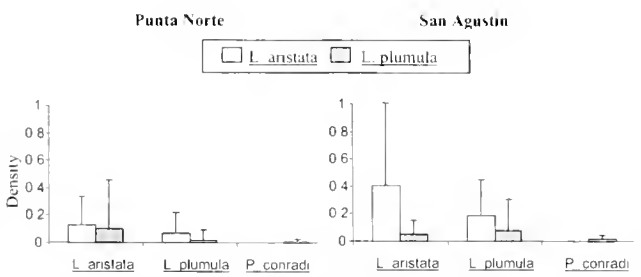

Figure 6. Burrowing clam density in abalone *Haliotis corrugata* and *H. fulgens* from the localities studied.

TABLE 3.

Comparison of total density of boring clams *Lithophaga* per abalone species.

	Punta Norte		**San Agustin**	
	L. aristata	***L. plumula***	***L. aristata***	***L. plumula***
H. corrugata	0.12	0.06	0.40	0.18
H. fulgens	0.10	0.02	0.05	0.07
Mann-Whitney U (*P*)	0.01	0.12	0.05	0.12

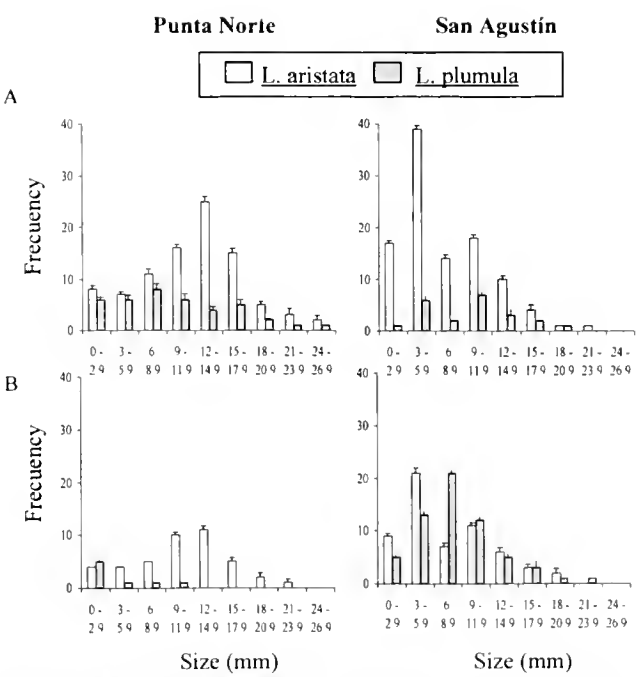

Figure 7. Mean size class distribution (±SE) for boring clams on the abalone *Haliotis corrugata* (A) and *H. fulgens* (B) from localities studied.

TABLE 4.

Spearman correlation coefficient (r) between boring clam density, area and age of *H. corrugata* and *H. fulgens* shells.

	Punta Norte				San Agustin			
	H. corrugata		*H. fulgens*		*H. corrugata*		*H. fulgens*	
	Area	Age	Area	Age	Area	Age	Area	Age
r	0.33	0.28	0.48	0.40	0.25	0.13	0.39	0.04
P	0.02	0.09	<0.00	0.02	0.26	0.62	0.17	0.88

The surface of the abalone shells was calculated using the weight of the aluminum molds obtained.

The age of the abalone was estimated by polishing the spire zone of each shell and counting the prismatic growth lines (dark lines) in the spire under a stereoscopic microscope, according to the method of Shepherd and Avalos-Borja (1997) and Shepherd et al. (1995). A correlation analysis was used to determine the association between the age and shell surface area of each abalone shell, as well as the intensity of clam infestation.

A comparison was made between the density of the infestation determined by extraction of the clams from the abalone shell and that observed on x-ray plates.

RESULTS

Three shell boring clams, *Lithophaga aristata* Dillwyn 1817, *Lithophaga plumula* Hanley 1843 (family Mytilidae) and *Penitella conradi* Valenciennes 1848 (family Pholadidae) (Fig. 3, Fig. 4, Fig. 5) were found infesting blue abalone shells. Only *Lithophaga aristata* and *Lithophaga plumula* infested the yellow abalone. The holes made by the three boring clams were similar and some reached the inner side of the host shell. A leathery layer of conchiolin of irregular and variable size often formed a pustule that occluded the perforation. Nacreous material occluded old perforations forming irregular half-pearls. Table 1, Table 2 and Fig. 6 show the mean, minimum and maximum boring clam densities and prevalence found per abalone species and locality. *Lithophaga aristata* and *L. plumula* were the more abundant boring clams; *Penitella conradi* was very scarce. Table 3 shows a comparison (Mann-Whitney U test) between density per abalone species and locality. In general, *Haliotis corrugata* had a higher density of boring clams than *Haliotis fulgens*.

The mean size distribution of boring clams is shown in Fig. 7. Distribution of mean size class at Punta Norte indicates that the size distribution of the burrowing clam population at that site is stable. In San Agustín, however, the mean size class distribution suggests a young population with recent recruitment.

In Punta Norte, older *Haliotis fulgens* individuals (with greater shell surface area) are infested with more boring clams per unit area than are younger abalone (with smaller shell surface area). The same pattern holds for *Haliotis corrugata* (Spearman correlation coefficient, Table 4).

Figure 8 shows the holes made by shell boring clams. Some clams remain in the holes and other epibionts, such as the boring sponge *Cliona* sp., make small holes in their shell. Only 81% of the infestation intensity obtained from direct count and extraction of the clams were revealed on X-ray plates, as boring clams on the edges of the abalone shell were not detected on the latter.

Figure 8. X-ray plate from *Haliotis fulgens*. Note the boring clams holes and boring clams inside (large arrow). Small dots indicate perforations produced by *Cliona* sp. or polychaetes (small arrow).

DISCUSSION

The differences in prevalence and density of infestation by boring clams between the abalone species from the same locality may be related to several factors, including age, shell area, abalone species, specific shell characteristics, vertical distribution of boring clams and particular environmental conditions. The total surface available for colonization by epibionts and the time spent underwater (age) by the substrate (abalone shell) are factors that determine the extent of colonization by benthic fauna. The depth distribution of the genus *Lithophaga* is similar to that of *Haliotis corrugata*, while *Penitella conradi* lives close to the lower intertidal zone where *Haliotis fulgens* is present (Soot-Ryen 1955, Keen & Coan 1974, Abbott & Haderlie 1980, Haderlie & Abbott 1980). A similar association was recorded in the red abalone *Haliotis rufescens* and black abalone *Haliotis cracherodii* in relation to the distribution of *P. conradi*, in California (Hansen 1970). The depth range of *H. rufescens*, which is primarily sublittoral, corresponds better to that of *P. conradi* than does that of the black abalone *H. cracherodii*.

The settlement of several mytilids, other bivalve species, and other invertebrates may be favored by filamentous and rugose substrates (Cáceres-Martínez et al. 1994). The rugosity of the yellow abalone's shell may thus present a better opportunity for settlement of mytilids and other invertebrates than the smoother shell of blue abalone. Shell chemistry may also influence boring clam settlement and penetration. Another consideration that could affect the density of epibionts on abalone shells is the differential growth rate of abalone from different localities. Some wild stocks of dwarf yellow abalone in Islas Benito have been recorded. These have a greater quantity of epibionts than those of normal growth (unpublished data).

Differences in boring clam prevalence and densities between the abalone from San Agustín and Punta Norte could be due to differential environmental conditions, since samples were taken on different dates (Punta Norte in November 1997, and San Agustin in June 1998). A direct comparison is thus difficult. Comparisons of growth rates and epibiont infestation between island abalone and mainland abalone in the area do not exist. The trend of more shell boring clams in older than in younger abalone, and in abalone with greater shell surface area than in those with smaller shell surface area must be investigated over a broad range of age and environment (mainland populations versus island populations).

To the best of our knowledge, this is the first record of the association of *Lithophaga aristata* and *L. plumula* with blue and yellow abalone. The genus *Lithophaga* has been widely recorded in association with corals (Scott 1986, Krumm & Jones 1993), submerged construction structures, sedimentary rocks (Kleeman 1994) and bivalves such as the pearl oyster (Doroudi 1996). *Penitella conradi* has been reported on shells of at least four abalone species on the Pacific coast, but most often in the red abalone (Haderlie & Abbott 1980).

Differences in the size distribution of burrowing clams from the studied localities indicate differences in recruitment and survival of these species. This may be associated with environmental conditions and collection dates. However, repeated monitoring of size class distribution is necessary to obtain information on the population dynamics of the burrowing clams. Moreover, it is necessary to investigate such environmental factors as currents, temperature and food availability to see if these are related to the presence and size class distribution of burrowing clams.

There are advantages and disadvantages to the two techniques used to determine the intensity of infestation by boring clams. The X-ray technique, although practical and non-destructive, does not allow identification of the boring clams and does not note the presence of all clams, missing those at the edges of the shell. On the other hand, extraction of the clams and direct counting is time consuming and shell destructive, although exact.

Economically, the results of this study highlight that it is possible for producers to detect certain zones that provide shells of different quality. It could thus enable managers to set regulations to protect abalone of these zones from the epibionts of other zones. For example, transplanting abalone from heavy burrowing clam-infested areas to those considered less infested could be controlled.

In conclusion, the yellow abalone had more boring clams, mainly of the genus *Lithophaga*, than the blue abalone. There is a trend of more boring clams in older and larger abalone (shell surface) than in younger and smallest abalone. More studies are needed to confirm the observed trend and to compare the density of boring clams between zones, and between mainland and island abalone populations. Additionally, studies investigating the possibility of managing different areas to obtain top quality abalone shells must be carried out.

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PARALYTIC SHELLFISH POISONING IN THE ABALONE *HALIOTIS MIDAE* ON THE WEST COAST OF SOUTH AFRICA

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ABSTRACT In April 1999, monitoring on two West Coast abalone farms provided evidence of the presence of Paralytic Shellfish Poisoning (PSP) toxins in the cultured abalone *Haliotis midae*. Subsequent analysis of wild animals collected from the West Coast also revealed the accumulation of PSP toxins in these gastropods. The toxicity of individual animals as measured by the AOAC mouse bioassay showed considerable variation, ranging from below the assay detection limit to a maximum of 1609 μg STXeq 100 g^{-1}. Initial observations found PSP toxins in abalone to be coincident with blooms of *Alexandrium catenella* indicating that this dinoflagellate was the probable cause of abalone toxicity. Subsequent detection by receptor binding assay, of toxicity in abalone on the South Coast, an area considered free of *A. catenella* blooms, casts some doubt as to the source of the toxins. The toxin composition in the abalone as determined by HPLC was dominated by STX, and differed significantly from the toxin profile of *A. catenella* and the co-occurring mussel, *Mytilus galloprovincialis*. Either these findings indicated a high capacity for biotransformation of PSP toxins by abalone or that *A. catenella* was not the source of the toxin. Investigation of the anatomical distribution of toxins revealed that they were not evenly distributed throughout the abalone tissues, but appeared to concentrate in outer layer tissue. The muscular foot made a disproportionately low contribution to the total toxin content of the mollusc, whereas the epipodial fringe, although comprising a small proportion of the abalone total weight, contributed substantially to the total toxin content. The epipodial fringe is typically included with the muscular foot as that part of the animal marketed for human consumption. The negative impacts of PSP contamination on abalone spawning and larval survival are presented and the findings of this study are compared to observations of PSP toxins in the abalone *Haliotis tuberculata* on the Galician coast. The inability of abalone to detoxify or depurate accumulated PSP toxins below the regulatory level threatens the future of the established abalone fishery and the newly developed aquaculture operations on the West Coast of South Africa.

KEY WORDS: Paralytic Shellfish Poisoning, saxitoxin, abalone, *Haliotis midae*, South Africa

INTRODUCTION

The abalone *Haliotis midae* forms one of the oldest fisheries on the South African coast, with present-day operations including recreational, subsistence, and commercial activities. During the 1990s, land-based farming of this species has also been under development and has recently attained commercial scale production. In April 1999, monitoring on two West Coast abalone farms provided evidence of the presence of Paralytic Shellfish Poisoning (PSP) toxins in cultured abalone. Subsequent analysis of wild animals also revealed the accumulation of PSP toxins in these gastropods.

The accumulation of PSP toxins typically occurs when toxic dinoflagellates are filtered from the water by bivalves such as clams, mussels, oysters, or scallops, which then accumulate the algal toxins to levels that are potentially lethal to humans or other consumers. Considerable progress has been achieved in the structural elucidation of PSP toxins. Following identification of the parent compound saxitoxin (STX), approximately two dozen naturally occurring derivatives have been described (Shimizu 1996), which vary greatly in their potency and are subject to interconversions and changes in relative proportions as they are transferred through the food chain. PSP occurs off the West Coast of South Africa and has always been attributed to the dinoflagellate *Alexandrium catenella* (Pitcher & Calder 2000), confirmed as the source of the toxin by Sapeika (1948). Although bivalve molluscs are the most common vectors of shellfish toxins, other vectors have been identified, including scavenging, predatory, and grazing gastropods (Shumway 1995). Abalone typically feed on kelp and other seaweeds. They are usually found attached to rocky substrates and feeding is accomplished by browsing on drifting seaweed. This feeding behavior and diet would indicate that abalone are unlikely candidates for the ingestion and accumulation of PSP toxins derived from toxic dinoflagellates such as *Alexandrium* spp., which primarily occur in the plankton.

This paper reports on investigations into the observations of PSP in abalone on the South African coast, including the incidence and distribution of toxic animals, the identification of the toxins, intrapopulation variation in toxicity, the anatomical distribution of toxins, and the depuration of toxins. The possible source and mode of uptake of toxins and the potential impact on spawning and recruitment success are also considered.

MATERIALS AND METHODS

Animals analyzed for this study were collected from five abalone farms, (Farms A–E) and from the field, at sites spread between the northern and southern farm locations (Fig. 1). All data presented here originate from animals collected between April 1999 and December 1999. A single wild animal collected in the vicinity of Jacobsbaai in April 1998 was also analyzed. The AOAC mouse bioassay was used in the routine analysis of PSP toxicity and a receptor-binding assay was utilized for comparative purposes. High performance liquid chromatography and mass spectrometry were used to determine and confirm, respectively, the

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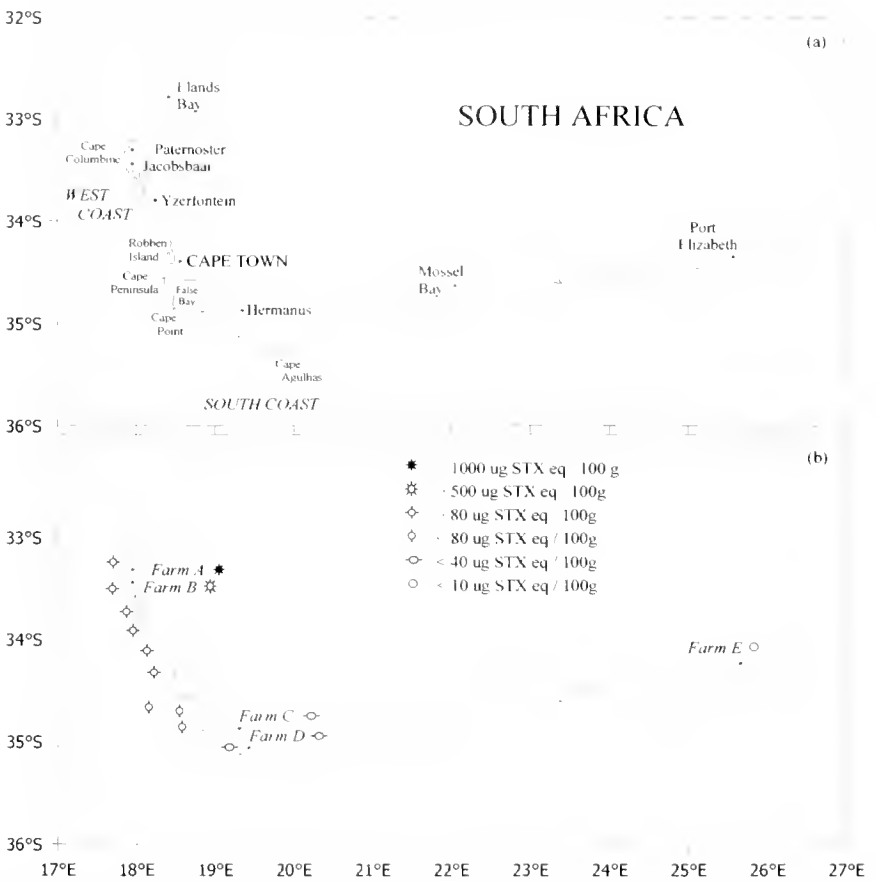

Figure 1. (a) Map depicting sample locations and (b) the occurrence of toxic abalone on the South African coast as indicated by the maximum toxicity recorded at each locality between April and December 1999 (toxicity >40 pg STX eq 100 g^{-1} was determined by the mouse bioassay; toxicity <40 pg STX eq 100 g^{-1} was determined by the receptor binding assay).

toxin composition in abalone. The toxin composition of the PSP-producing dinoflagellate, *A. catenella*, and the mussel, *Mytilus galloprovincialis*, collected at Elands Bay during March 1999 were also determined for comparative purposes.

Intrapopulation variability in toxicity was investigated by analysis of individual animals of the same size cultured under similar conditions or collected from the same locality in the wild. In most instances, the entire animal, once removed from the shell, was used in the analysis. In other cases, toxin analysis was carried out on specific body parts (e.g., the viscera, foot, epipodial fringe, digestive gland, gonad, mantle, or gills) to examine the anatomical distribution of toxins. Foot and epipodial fringe tissues were also analyzed following removal of epithelial tissue by scrubbing. Typically, analyses were performed on individual abalone, but in some cases, animals were pooled for analysis.

In assessing the rate of toxin depuration, 60 cultured animals were removed from Farm B on 13 May 1999 and 60 wild animals were removed from Paternoster on 29 June 1999, and placed under controlled conditions in a Cape Town laboratory. The animals were maintained on a diet of kelp in flow-through tanks supplied with filtered (18 μm) seawater. Abalone were periodically sacrificed to assess the rate of depuration. The mouse bioassay was used to measure toxicity and two animals were sacrificed for each assay in order to reduce the effects of intrapopulation variability in toxicity.

In an attempt to ascertain the source of the toxins, time-series data on the incidence of the PSP-producing dinoflagellate, *A. catenella*, at Elands Bay on the West Coast are presented. Data on the distribution of PSP toxin-contaminated bivalves, as monitored during the period 1989–1999 are also presented. These data were collected as part of a Harmful Algal Bloom monitoring program detailed by Pitcher and Calder (2000). The distribution of toxic shellfish is compared to the distribution of abalone catches, determined by the Total Allowable Catch allocations, which generally reflect the distribution of abalone (R. Tarr pers. comm).

When PSP contamination of cultured abalone was initially detected, problems with spawning and larval survival were experienced on affected West Coast farms. Samples of eggs and larvae from these farms were examined by light and scanning electron microscopy and compared to normal eggs and larvae of the same age from a farm on the South Coast. Samples of eggs were collected at spawning, at fertilization, and at regular intervals until hatching. Samples of larvae were collected at regular intervals from hatching until settlement. All samples were taken in duplicate, one sample placed in 4% gluteraldehyde in 0.2 M sodium cacodylate buffer and the other in Davidson's fixative (Austin and Austin 1989). Animals fixed in gluteraldehyde were processed for scanning electron microscopy. Samples were rinsed twice in sodium cacodylate buffer, after which they were dehydrated through an ascending series of ethanol. The samples were then critical point dried from 100% ethanol through liquid carbon dioxide in a Polaron Critical Point Drier. Dried samples were mounted onto viewing stubs and sputter coated with gold. Samples were viewed at 3 to 8 kV accelerating voltage in a Hitachi S-2500 Scanning

Electron Microscope. Animals fixed in either gluteraldehyde or Davidson's fixative were processed for light microscopy. Samples were rinsed twice in distilled water to remove adhering detritus. A modified double embedding technique (Austin & Austin 1989) was used prior to processing routinely for light microscopy. Sections were cut at 6 μm thickness and stained with haematoxylin and eosin.

The following procedures were adhered to in the analysis of PSP toxins:

Mouse Bioassay

Net toxicity was measured by the standard AOAC mouse bioassay (Association of Official Analytical Chemists, AOAC 1990), the method adopted worldwide to monitor the safety of shellfish PSP toxin levels for human consumption. Acidic aqueous extraction of the tissue in 0.1 M hydrochloric acid (heated to 100° C) was followed by intraperitoneal injection of 1 ml of the extract into each of three standardized mice. The time from initial injection to mouse death was recorded and the toxicity determined from Sommer's table.

HPLC-FD

High performance liquid chromatography with fluorescence detection (HPLC-FD) was used to determine the concentration of individual PSP toxins (Franco & Fernández -Vila 1993). The separation of toxins was carried out in a 5 μm Lichrospher 100 RP-18 column (125 × 4 mm i.d.). Two isocratic elutions were used, the first, for the separation of the carbamate toxins, neoSTX, dcSTX and STX, and the second for the separation of the GTX toxins. The first eluent was 1.5 mM octane sulfonate in 10 mM ammonium phosphate buffer (pH 7.2) plus 6% acetonitrile, at a rate of flow of 1 mL min^{-1}. The second eluent was 2 mM octane sulfonate in 10 mM ammonium phosphate buffer (pH 7), at a rate of flow of 0.8 mL min^{-1}. The detector was a Waters 474 spectrofluorimeter, set at 330 nm ex., 390 nm em. Millennium software was used for recording and integrating peaks. Two pumps were used for delivering postcolumn reagents. The postcolumn reaction was performed in a Teflon coil (10 m × 0.5 mm i.d) at 65° C. The first reagent was the oxidant, a solution of 7 mM periodic acid in 50 mM sodium phosphate (pH 9), and the second the acidifiant, a 0.5 M solution of acetic acid. Both were delivered at a rate of flow of 0.4 mL min^{-1}. The toxin reference standards for dcSTX and STX were supplied by the project EUR 18318 of the European Commission. The remaining reference standards for neoSTX and GTX_{1-4} were purchased from the National Research Council of Canada. The C_s, GTX_5 and GTX_6 toxins were identified and quantified by mild acid hydrolysis of the sample with an equal volume of 0.4 M HCl at 100°C for 15 min. This process transforms C_1 to GTX_2, C_2 to GTX_3, C_3 to GTX_1, C_4 to GTX_4, GTX_5 to STX and GTX_6 to neoSTX. The values of toxicity were expressed in μg STX eq 100 g^{-1} tissue thereby enabling comparison of HPLC and mouse bioassays. Values in μg toxin g^{-1} were derived from the sum of the concentrations of all toxins detected by HPLC. These values were converted to μg STX eq 100 g^{-1} by means of molar specific conversion factors inferred from the values of specific toxicity given by Oshima (1995).

Confirmation of the presence of STX was performed by mass spectrometry with an ion-trap mass spectrometer model LCQ (Finnigan, ThermoQuest, USA). Off-line "nanospray" ionization was carried out using disposable gold-coated capillary probes, as described previously by Marina et al. (1999). The specific detection of toxins at high sensitivities was performed by examining for the appearance of daughter ions produced by the fragmentation of the corresponding precursor ions.

Receptor Binding Assay

The receptor binding assay used in the detection of PSP toxins exploits the highly specific interaction of these compounds with their biological receptor, the voltage-dependent sodium channel, and is therefore based on functional activity (Doucette et al. 1997, Powell & Doucette [In Press]). This assay has been compared with mouse bioassay-based toxicities of AOAC extracts of various bivalve molluscs and results between methods agreed closely (Doucette et al. 1997). The assay protocol and calculations of sample values used herein follow those described by Doucette et al. (1997) and modified by Powell and Doucette (In Press). Briefly, the receptor assay was performed in a microplate filtration format and involved the incubation of a rat brain synaptosome preparation containing the receptors, with [^{3}H]-STX and unlabeled PSP toxins contained in a standard or sample. After removal of unbound toxin by washing, the remaining bound radioactivity (i.e., [^{3}H]-STX), which is inversely and quantitatively related to the concentration of PSP toxins in a sample, was determined by scintillation counting on a Wallac MicroBeta model 1450. Toxin concentrations were expressed in terms of μg STX eq 100 g^{-1}.

RESULTS AND DISCUSSION

Incidence and Distribution of Toxic Abalone

In April 1999 monitoring of abalone at Farm B, for PSP toxins by means of the mouse bioassay, yielded positive results. Subsequent tests at the nearby Farm A were also positive and many of these animals were reported to be paralyzed, in that they were no longer able to attach to a substrate and were unable to right themselves. Brood stock were amongst the animals affected and some mortalities were recorded. At this time, divers reported the presence of a large number of detached or paralyzed abalone in the kelp beds in the vicinity of Paternoster and analysis of these animals also indicated the presence of PSP toxins. Subsequent to these initial findings, approximately 300 abalone, both cultured and wild, from five abalone farms and from several sites in the field have been analyzed by mouse bioassay. Although toxin concentrations varied considerably at each locality, the highest toxin concentrations were recorded on the northernmost farm, Farm A, where the single highest value was 1609 μg STX eq 100 g^{-1}. Toxin concentrations generally decreased southwards and the Cape Peninsula was the southern limit of toxic abalone as detected by the mouse bioassay (Fig. 1).

On the two West Coast farms toxin concentrations exceeded the regulatory level of 80 μg STX eq 100 g^{-1} in the majority of animals, particularly on Farm A (Fig. 2a, b). Although toxins were detected in nearly all the wild animals tested on the West Coast, toxin concentrations were less than the regulatory limit in the majority of these animals (Fig. 2c). Of all the abalone tested on the South Coast by mouse bioassay, toxicity was detected only in animals collected on the western shores of False Bay (Fig. 2d). However, analysis of abalone from Farms C, D and E by means of the more sensitive receptor binding assay revealed the presence of PSP toxins at concentrations below those detectable by the mouse bioassay (Fig. 1b). Assessment of all toxin analyses indicated a

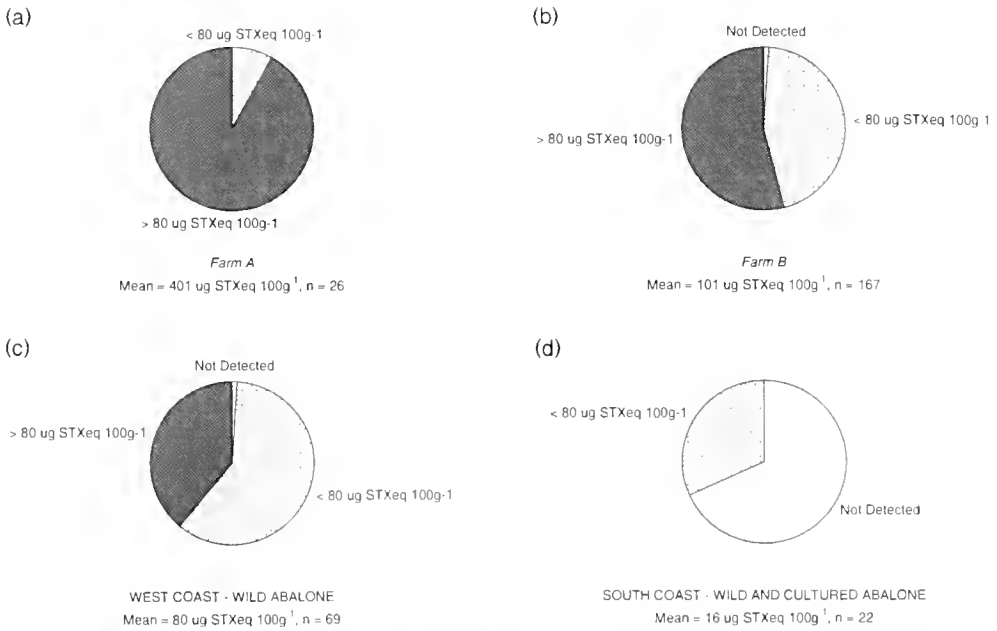

Figure 2. The incidence of toxic abalone as detected by the mouse bioassay on (a) *Farm A* (b) *Farm B* (c) from wild animals collected on the West Coast, and (d) from both cultured and wild animals collected on the South Coast.

geographical gradient in abalone toxicity, toxin concentrations decreasing from north to south.

Analysis of a single wild abalone from Jacobsbaai collected in April 1998 by means of the mouse bioassay provided a negative result. Although not conclusive, this single sample suggests that PSP toxins were not present in abalone, at concentrations detectable by the mouse bioassay, during the previous year.

Toxin Composition

As the mouse bioassay is a non-specific test and owing to the fact that abalone are unlikely candidates for the accumulation of PSP toxins, analysis by means of HPLC and mass spectrometry was required to identify and confirm the presence of PSP toxins. The toxin composition of abalone as determined by HPLC was rather unusual in that it was dominated by a single toxin, notably STX (Fig. 3a). The presence of STX was confirmed by mass spectrometry by the appearance of daughter ions (282 DA) produced by the fragmentation of the corresponding precursor ions (300 DA).

The specific toxicity and toxin composition of the PSP-producing dinoflagellate, *Alexandrium catenella*, present in bloom proportions in Elands Bay during March 1999, were also investigated. Different dinoflagellate strains vary greatly in their specific toxicity depending on environmental and growth conditions, and the single measurement of 1.75 pg STX eq $cell^{-1}$ classify this dinoflagellate as a strain of low toxicity. The relative proportion of various PSP derivatives is a conservative property for a given isolate (Cembella et al. 1987) and the blooms in Elands Bay were found to be characterized by a high proportion of the less toxic N-sulfocarbamoyl toxins (C_1 and C_2) and moderate proportions of the more potent carbamate (STX and GTX_4) and decarbamoyl toxins ($deGTX_3$) (Fig. 3b).

Simultaneous collection and analysis of the mussel, *Mytilus galloprovincialis*, from Elands Bay during March 1999 provided toxicity values of 1 610 and 60 940 μg STX eq 100 g^{-1} as determined by the mouse bioassay and HPLC, respectively. A significant difference between the toxin profile of the causative dinoflagellate and the contaminated bivalve indicated a high degree of toxin transformation (Fig. 3c). It is well known that the toxin composition in bivalve tissues can differ significantly from that of the toxic dinoflagellates ingested (Bricelj & Shumway 1998) and as is often the case with bivalves, the mussels were found to contain reduced proportions of N-sulfocarbamoyl toxins (C_1 and C_2) and higher proportions of the carbamate toxins (neoSTX and STX). This observation is common when the algae ingested are rich in N-sulfocarbamoyl toxins which are more labile than other toxins and therefore more likely to be transformed to their more toxic carbamate analogs (Hall & Reichardt 1984). Although the precise in vivo mechanism of conversion between N-sulfocarbamoyl and carbamate toxins in shellfish has not been determined, it may lead to an increase in net toxicity (Bricelj & Shumway 1998). The dramatically different toxin profile of *H. midae* compared to *A. catenella*, indicates either a very high capacity for biotransformation of PSP toxins by abalone or that *A. catenella* was not the source of the toxin.

Toxicity as measured by HPLC was notably higher than that measured by the mouse bioassay where y and x are toxicity measured by HPLC and mouse bioassay respectively,

$$y = 2.34x + 340.7,\ F = 12.1,\ r^2 = 0.57,\ p < 0.007$$

Intrapopulation Variation in Toxicity

Toxicity as measured by the mouse bioassay showed considerable variation between individual animals, exceeding that accounted for by imprecision in the mouse bioassay (ca. +/– 20%, Adams & Furfari 1984). For example, on Farm A, the measured values of toxicity for whole animals ranged from 63–1609 μg STX eq 100 g^{-1}. An understanding of the causes of this high intrapopulation variation in toxicity is crucial in the design of monitoring programs for the assurance of seafood safety (Bricelj & Shumway 1998).

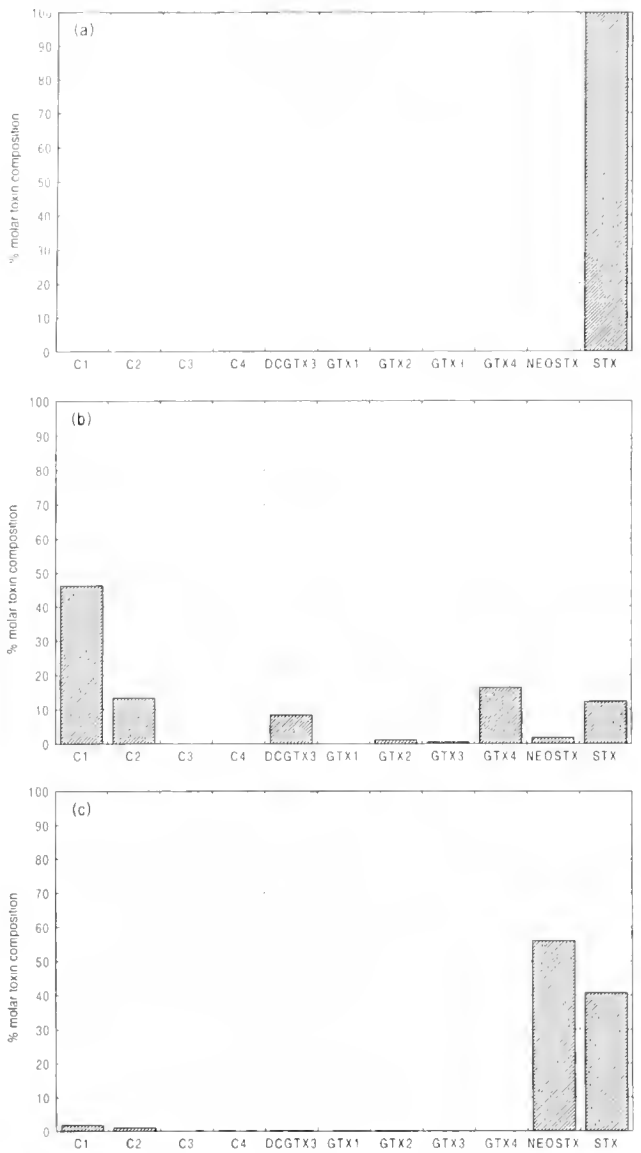

Figure 3. Toxin composition of (a) cultured and wild abalone *Haliotis midae*, (b) a phytoplankton sample with a high proportion of cells of *Alexandrium catenella* and (c) the bivalve *Mytilus galloprovincialis* (the phytoplankton and bivalve samples were collected during March in the vicinity of Elands Bay).

In this study, animals of a similar size and subjected to similar conditions of exposure to PSP toxins, such as farmed animals from a single basket or wild animals from a particular region, demonstrated marked variability in toxicity (Fig. 4). The toxicity of 10 animals (size: 55–60 mm) sampled from a single basket on Farm B, ranged more than 5-fold from 57–307 μg STX eq 100 g^{-1}. Similarly, the toxicity of 10 animals (size: all 30 mm) sampled from a single basket on Farm A, also ranged 5-fold from 77–383 μg STX eq 100 g^{-1}. The toxicity of 52 animals collected in the vicinity of Robben Island (size: 115–170 mm) ranged more than 10-fold from 29–314 μg STX eq 100 g^{-1}. This variability prevented the identification of any trends in toxicity associated with individual differences, such as body size, which are considered to influence toxin accumulation rates.

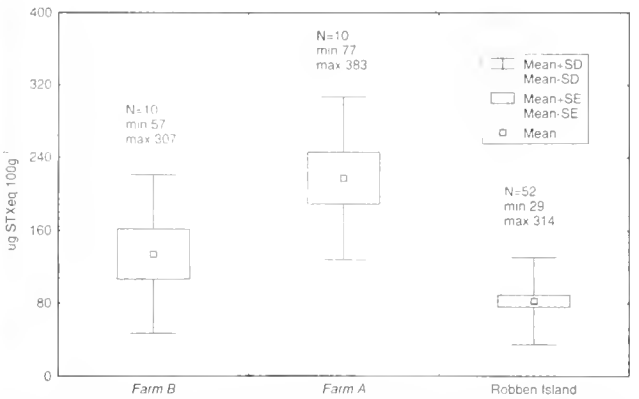

Figure 4. Intrapopulation variability in toxicity in abalone tested from a single basket at each of *Farms A* and *B*, and from abalone harvested in the vicinity of Robben Island.

Anatomical Distribution of Toxins

Paralytic shellfish toxins are not evenly distributed throughout shellfish tissues, resulting in pronounced differences in the absolute toxicities of individual tissues. The contribution of each tissue to the total toxin body burden is therefore a function of both its absolute toxicity and relative weight contribution.

Discarding of those tissues that selectively sequester PSP toxins (e.g. evisceration) may, in some cases, provide an effective tool to reduce the risk of PSP (Bricelj & Shumway 1998). In many instances only the foot of the abalone is marketed for human consumption, therefore the anatomical distribution of toxins is of considerable interest and importance. This study has revealed that toxins are not evenly distributed throughout the tissues of abalone (Fig. 5). Initial investigations divided the animals into three components, the viscera, foot, and epipodial fringe. Results indicated moderate toxicity in both the viscera and foot with dramatically higher levels of toxin in the epipodial fringe, a body component with a high surface area. Therefore, the muscular foot of abalone, despite contributing substantially to the total weight of the abalone, makes a disproportionately low contribution to the total toxin content of the animal. The epipodial fringe, while making only a small contribution to the total weight of the abalone, contributes substantially to the total toxin content and is typically included with the muscular foot in that part of the animal marketed for human consumption. The removal of epithelial tissue by scrubbing both the foot and epipodial fringe dramatically lowered the toxin levels in these tissues, suggesting that the toxins were concentrated in the epithelium (Fig. 5a). Scrubbing could therefore provide a possible means of reducing the levels of toxin prior to marketing the product for consumption.

Further studies included examination of gill, mantle, digestive gland, and gonad tissue (Fig. 5b). These results varied somewhat from the initial investigations. Very high toxin levels were evident in the gill tissue, again indicating the accumulation of toxins in outer layer tissue. The toxicity of the digestive gland was also particularly high. Although toxin levels in the epipodial fringe were substantially less than those measured in the initial experiments, they were significantly higher than those in the foot and gonad. The capacity for in vivo biotransformation and the selective retention of individual PSP toxins, are major determinants of the differences in toxicity among tissues. However, establishment of

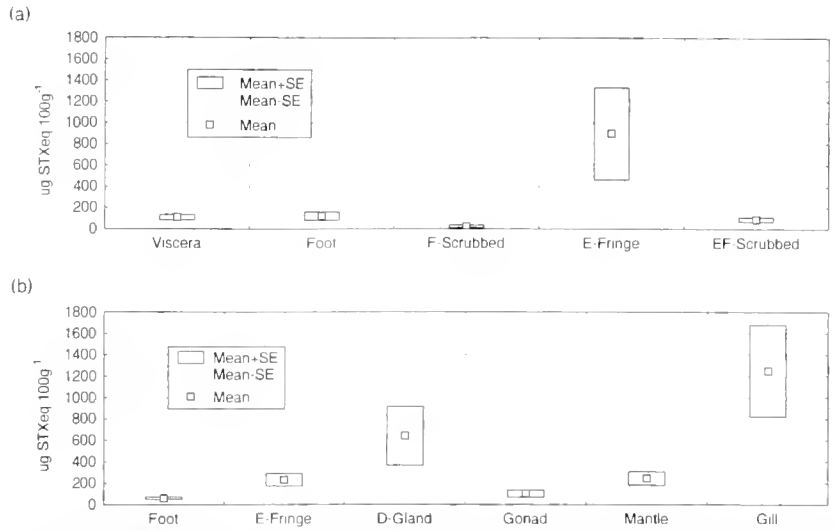

Figure 5. Anatomical distribution of toxins as determined in (a) the viscera, foot, and epipodial fringe (toxin concentrations were also determined following removal of epithelial tissue from the foot and epipodial fringe by scrubbing (n = 12)) and (b) the foot, epipodial fringe, digestive gland, gonad, mantle, and gill tissue (n = 10).

the source and mode of uptake of the toxins in abalone is required to understand the distribution of toxins and how they may change over time.

Depuration of Toxins

In order to manage the problem of PSP in abalone it is important to establish the rate of depuration of toxins, as shellfish are known to vary markedly in their ability to detoxify accumulated toxins. Cultured and wild abalone were placed under controlled aquarium conditions and periodically sacrificed and analyzed for toxins in order to assess the rate of detoxification (Fig. 6). The results obtained over a 7-month period indicate that the prolonged retention of toxins is a characteristic of abalone. During this period, measures of toxicity ranged from below the regulatory level to 853 μg STX eq 100 g^{-1} with no apparent declining trend, thus reflecting only the high intrapopulation variation in measured toxin levels.

Incidence of Alexandrium catenella *and PSP*

The only known source of PSP toxins in the Benguela is the dinoflagellate, *A. catenella*, which is usually associated with red tide on the West Coast of South Africa (Pitcher & Calder 2000). A seven year time-series obtained from daily monitoring at Elands Bay provides an indication of the interannual variability in the incidence of *A. catenella* in the plankton on the West Coast (Fig. 7). Although *A. catenella* is typically observed each year as a component of the plankton, usually in the latter months of summer, the detection of PSP toxins in abalone for the first time appears to have coincided with particularly high concentrations of *A. catenella*. Furthermore, at the time at which toxins were first detected in abalone on Farm B, examination of water samples from the farm revealed an abundance of *A. catenella*.

Blooms of this dinoflagellate are common north of Cape Columbine and are usually advected southwards, often as a flood event, during late summer and autumn. Blooms typically do not extend beyond Cape Point onto the South Coast (Pitcher & Calder 2000). Consequently the highest incidence of PSP is observed north of Cape Columbine while PSP has not been recorded east of Cape Point (Fig. 8a). Initial observations of PSP toxins in abalone therefore coincided temporally and spatially with blooms of *A. catenella*, indicating that this dinoflagellate was the probable cause of abalone toxicity. Subsequent detection, however, of low concentrations of PSP toxins in abalone on Farms C, D and E, on the

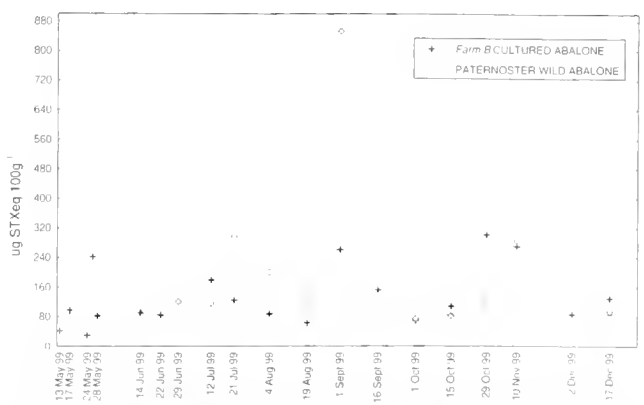

Figure 6. A time series of toxin concentrations in abalone removed from *Farm B* and from the wild at Paternoster and maintained under controlled laboratory conditions.

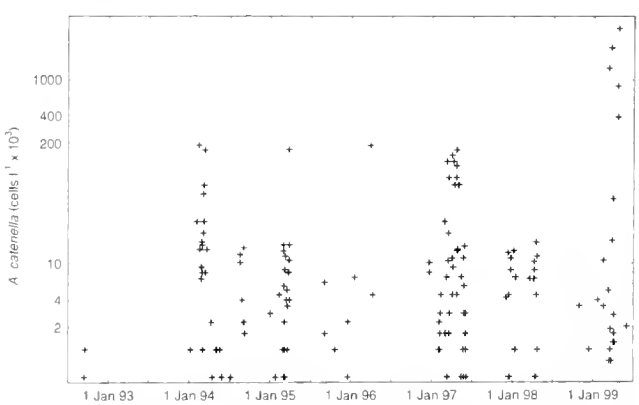

Figure 7. Daily time series of *Alexandrium catenella* concentrations at Elands Bay for the period 1 July 1993 – 30 June 1999.

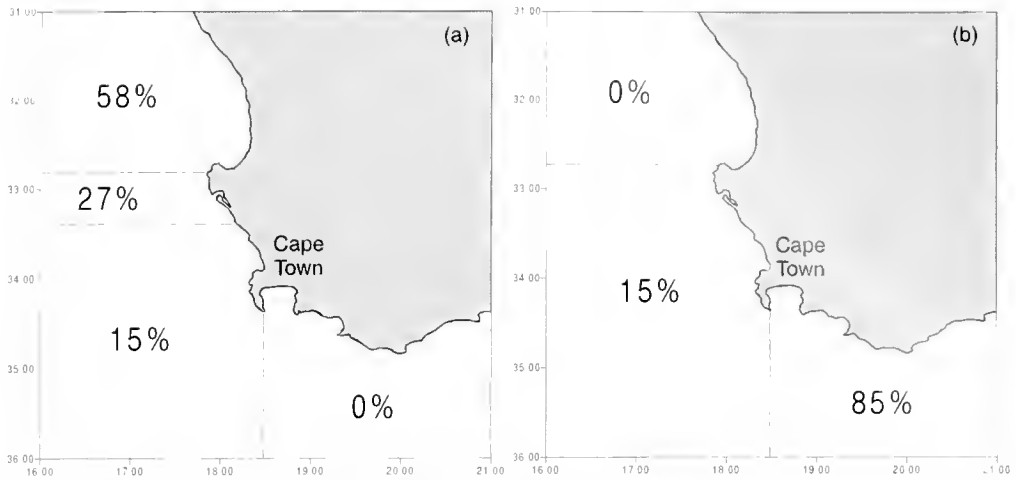

Figure 8. (a) The incidence of PSP-contaminated mussels, *Mytilus galloprovincialis*, as monitored during the period 1989–1999 (as % of the total samples in which PSP toxins were detected by means of the mouse bioassay) and (b) the distribution of abalone catches.

South Coast, an area considered to be free of blooms of *A. catenella*, casts some doubt as to the source of PSP toxins in abalone at least in this region.

Potential Ecological Impact

The contamination of bivalves is usually facilitated by their relative insensitivity to PSP toxins. However, it has become apparent that harmful algae may compromise survival and growth in some bivalve populations (Shumway 1990), although little is known about the ecological effects PSP toxins may have on field populations. Results of this study indicate that within the abalone fishery, recruitment may be severely influenced by PSP.

The contamination of abalone with high levels of PSP toxins on Farm A was accompanied by clinical signs of paralysis in certain animals, including brood stock. Animals dropped off the sides of holding tanks and were unable to right themselves, and some mortalities were experienced. Histological examination of affected animals showed no significant abnormalities. Brood stock that recovered resumed spawning, but viable larvae were not produced. Light and scanning electron microscopy of eggs and larvae from Farm A showed irregular division of eggs and the formation of grossly abnormal larvae (Fig. 9). The larvae were characterized by irregular shapes and the appearance of cilia in clumps over their entire surface. Although most eggs containing such larvae hatched, the larvae were unable to swim and did not develop further. Problems with larval development persisted for several months. It is not possible to say whether the larval abnormalities were directly related to PSP contamination. It is likely that the reproductive problems were caused by the deterioration in brood stock health and that the effect of the toxin on larval development is therefore only indirect. Similar, larval abnormalities could probably be caused by other brood stock stressors, but further research is needed to investigate this possibility.

In comparing the distribution of PSP toxin-contaminated mussels (Fig. 8a) to the distribution of abalone catches (R. Tarr, pers. comm) which generally reflect the distribution of abalone (Fig. 8b), we find them to be inversely distributed. That is, abalone are absent from areas where there is a high incidence of PSP, but abundant in areas not prone to PSP. Without any obvious physiological or ecological factors determining the northern limit to the distribution of abalone, these results suggest that the absence of abalone north of Columbine may be determined by the higher incidence of PSP toxins in that region.

A Comparison to the Observations of PSP Toxins in Haliotis tuberculata *on the Galician Coast*

The only other occurrence of PSP toxins in abalone has been reported in the species *Haliotis tuberculata* on the Galician coast of Spain. In 1991 Martinez et al. (1993) detected PSP toxicity in samples of abalone collected in the Ria de Arousa, and the Galician Coast was closed to the harvesting of abalone in 1993 as toxin levels exceeded the regulatory limit (Bravo et al. 1996). A number of comparisons may be made between the observations of PSP toxins in abalone on the Galician and South African coasts. The single greatest difference was in the toxin composition profile Fig. 10). Whereas *H. midae* was dominated by STX, *H. tuberculata* was dominated by decarbamoylsaxitoxin (dcSTX) with a small proportion of STX (Nagashima et al. 1995, Bravo et al. 1999). Similarities included the observation of highest toxicity in the epithelium of the *H. tuberculata* foot (Bravo et al. 1999), with the absolute toxicity of the epithelium being more than 300-fold greater than that of the gut or muscle. Another similarity was the slow depuration of toxins, as Bravo et al. (1996) observed no depuration of toxins in *H. tuberculata* maintained under controlled laboratory conditions for 3 mo.

As is the case in *H. midae*, the source or origin of toxins in *H. tuberculata* remains uncertain, as the geographical range of the toxic abalone does not coincide exactly with the PSP-producing dinoflagellates known in the region. On the Galician Coast, no significant differences have been observed in the toxicity of abalone from different locations, despite a higher incidence of toxic dinoflagellates on the central and southern coasts. Since abalone attach to rocky substrates and browse on kelp and other seaweeds, the toxins may originate from these seaweeds. Other potential sources may include bacteria and bluegreen algae. If toxic dinoflagellates are the source of toxins in abalone, the mode of

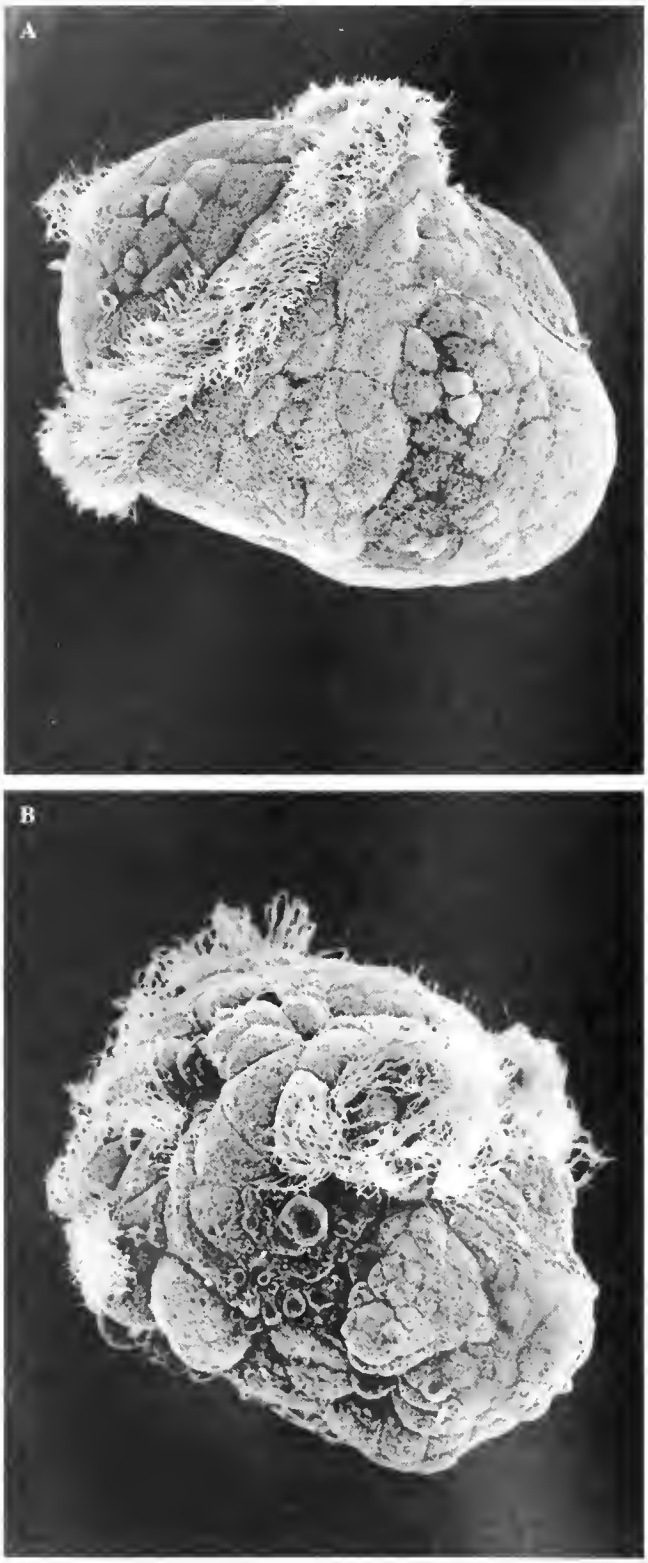

Figure 9. (a) A normal newly hatched larva known as a trochophore stage and (b) an abalone larva produced by PSP-contaminated broodstock. The normal larva is able to swim and remains planktonic for approximately five days. It is regular in shape, possesses a clearly defined protostomal tuft and a continuous and complete velum.

uptake remains to be determined. It is possible that settled vegetative cells or cysts may be taken up during feeding and observations in culture have indicated a high incidence of *A. catenella* cells associated with the mucilage layer of kelp fronds. The production of extracellular toxins by *A. catenella* may also serve as a possible source of PSP toxins and the adsorption/uptake of these toxins by abalone may provide an explanation for the accumulation of toxins in outer layer tissue.

The observations of PSP toxins in abalone also show several similarities to those made on the butter clam *Saxidomas giganteus*. Toxins were, as in *H. midae*, preferentially accumulated as STX in the outer surface of the siphons. STX was accumulated in clams feeding on an *Alexandrium* isolate containing GTX and neoSTX but no STX, and STX accumulated in the siphon only after the depuration of GTX and neoSTX (Beitler & Liston 1990). Although immunohistochemical studies showed the location of STX to be in the columnar epithelium (Smolowitz & Doucette 1995), the mechanism of tissue-specific retention of PSP toxins remains to be elucidated. Long-term retention, of high levels of STX has also been reported, typically taking several months to years to detoxify below the regulatory limit (Quayle 1969, Beitler & Liston 1990). Price and Lee (1971), and Price and Lee (1972) suggested that pH-dependent binding of STX to the melanin-containing tissues of the siphon was a mechanism of toxin retention in this species and Kvitek (1991), and Kvitek (1993) speculated that toxin compartmentalization in butter clams had evolved as a chemical defense mechanism to reduce predation on clams. A similar mechanism of STX retention may exist in abalone which also possess melanin-containing tissue (Fox 1983). However, recent investigations by Bravo et al. (Submitted) have indicated that the toxins are associated with secretory cells of the epithelium and not with cells containing granules of melanin-like pigment.

CONCLUSION

The findings of this study support the need for the inclusion of non-filter feeding molluscs in routine monitoring programs. The presence of PSP toxins in abalone now threatens the future of the abalone fishery and the newly developed aquaculture operations on the West Coast of South Africa. This threat is made more ominous by the slow depuration of toxins to below the regulatory level and by our limited understanding of the causes of the problem. Priority areas of research should therefore focus on identification of the origin and establishment of the mode of uptake of the toxins. The concentration of toxins in the epithelium may provide the possibility for the development of a fairly simple treatment to reduce the toxicity to below the regulatory limit in the marketable product. Management of the problem of PSP toxins in abalone by such means needs to be explored further.

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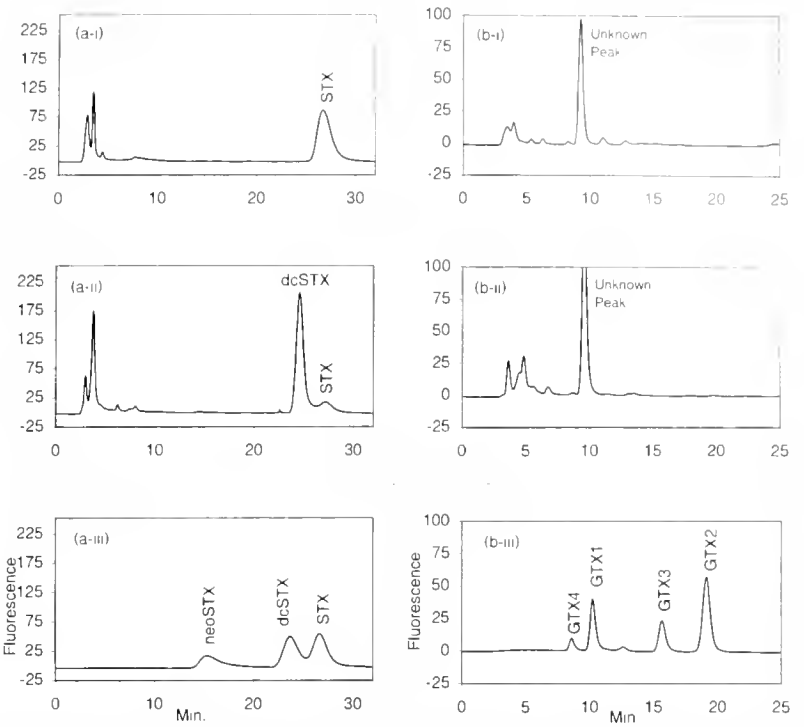

Figure 10. A comparison of toxin profiles in abalone on the South African and Galician coasts. Chromatograms of the (a) primary and (b) secondary isocratic elutions of (i) *Haliotis midae* (ii) *Haliotis tuberculata* and (iii) the toxin standards.

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COVER PHOTO: A group of large, adult "perlemoen" (South African abalone: *Haliotis midae*) against a backdrop of their primary food source, the kelp *Ecklonia maxima.* Photographed in a typical exposed habitat in 4 m water depth, Betty's Bay, South Africa, November 1988. (Rob Tarr)

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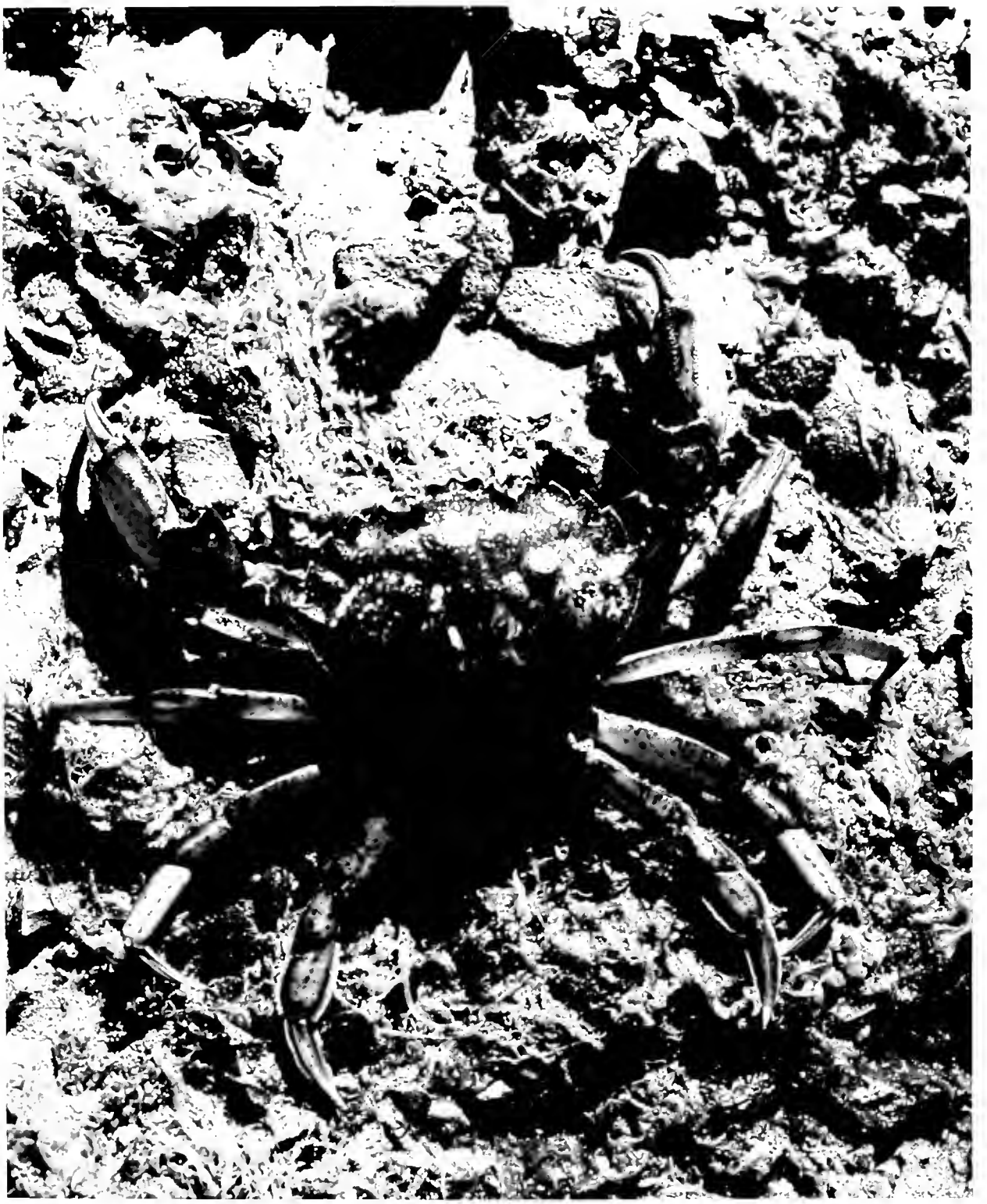

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FIELD IDENTIFICATION OF THE EUROPEAN GREEN CRAB SPECIES: *CARCINUS MAENAS* AND *CARCINUS AESTUARII*

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ABSTRACT Adults of the global invaders, *Carcinus maenas* and *C. aestuarii*, can generally be distinguished in the field by three diagnostic characteristics: the shape of the copulatory appendages (pleopods) in the male, the shape of the frontal area between the eyes, and the carapace width to length ratio. The pleopods of male *C. maenas* are crescent-shaped and curve outward with the center of the crescents touching; those of *C. aestuarii* are straight and parallel to one another. The frontal area of *C. maenas* does not protrude and is bordered by three scalloped-shaped lobes with distinct bumps. The frontal area of *C. aestuarii* is flat without distinct bumps and protrudes beyond the eyes. The carapace width to length ratio of adult crabs is typically >1.29 for *C. maenas* and <1.27 for *C. aestuarii*.

KEY WORDS: *Carcinus maenas*, *Carcinus aestuarii*, sibling species, invasive species, green crab

INTRODUCTION

The European green crab, a native to Europe and North Africa, is receiving much attention lately because of its success as a global invader. Established breeding populations outside its native range are currently found in Japan, South Africa, Australia, Tasmania, and on both the east and west coasts of North America (Le Roux et al. 1990, Cohen et al. 1995, Grosholz & Ruiz 1995, Furota et al. 1999).

Two forms of the green crab are now recognized: the Atlantic *Carcinus maenas* (Linnaeus, 1758) and the Mediterranean *C. aestuarii*, Nardo, 1847 (old name *Carcinus mediterraneus* Czerniavsky, 1884) (Demeusy & Veillet 1953, Demeusy 1958, Holthuis & Gottlieb 1958, Almaça 1961, Zariquiey Alvarez 1968, Bulheim & Bahns 1996, Geller et al. 1997). The Strait of Gibraltar, with its high sill, acts as a partial barrier to larval and gene exchange. Consequently, Mediterranean populations of many marine organisms, including the green crab, diverged from their Atlantic counterparts (Demeusy & Veillet 1953, Barsotti & Meluzzi 1968, Almaça 1989, Quesada et al.1995, Saavedra et al. 1995, Bulnheim & Bahns 1996, d'Udekem d'Acoz 1999). There is evidence, however, of some mixing of *Carcinus* populations near the Strait of Gibraltar (Almaça 1961, Clark et al. 2001, Armand Kuris, unpubl. observations).

Within the last two centuries, *Carcinus* has been accidentally introduced into several regions outside its native range as a result of maritime commerce and ballast transport: *C. maenas* to the east and west coast of North America, Australia, Tasmania, and South Africa; and *C. aestuarii* to Japan (Almaça 1962, Geller et al. 1997). Since *Carcinus* spp. tolerate air exposure, starvation, and wide ranges in temperature and salinity, they are well adapted to survive ocean voyages and plane rides. Furthermore, the free-swimming larvae have the potential to survive within the ballast tanks of cargo ships. Once released into a new environment, these ecological generalists can subsist on a variety of food organisms, including marsh vegetation, algae, crustaceans, mollusks, and fish (Cohen et al. 1995). Under favorable conditions, both species can reach sexual maturity within 1 y (Grosholz & Ruiz 1995, Furota et al. 1999, Behrens Yamada & Hunt 2000). The planktonic larvae produced by colonists can travel on ocean currents and "seed" new breeding populations elsewhere (Behrens Yamada & Hunt 2000). Marine scientists, resource managers, shellfish growers, and members of the general public are concerned that these invaders may adversely affect marine communities by altering food webs, disturbing habitats, displacing native species, and preying on commercially important clams, mussels, oysters, and juvenile native crabs.

When a green crab is sighted in a new geographical area, it is important to rapidly confirm its identity, its source, and its mode of introduction. Once a vector has been identified, steps can be taken to prevent further individuals from arriving. If the numbers of a new invader can be kept below a critical threshold, a self-perpetuating population may not establish itself. Time is thus paramount in identifying the invader's origin. While it may be possible to identify the most likely source of a new green crab invader with genetic tools (Bulnheim & Bahns 1996, Geller et al. 1997, Bagley & Geller 2000), these procedures take time and resources to implement. We propose that a fast, simple field identification method be used to identify which *Carcinus* species is actually invading.

Green crabs can be distinguished from other crabs in Europe and North America by their fan-like shaped carapace, five prominent, sharp anterio-lateral teeth behind the eyes, and three lobes between the eye sockets (Fig. 1; Crothers & Crothers 1988). Distinguishing the Atlantic from the Mediterranean species, however, takes closer inspection. Various studies have focused on green crab species identification, but these references are not readily available to most English-speaking scientists and resource managers because they are dated and/or published in Spanish, French, and Portuguese journals. Our goals are to list all the distinguishing characteristics described in the literature and to evaluate the usefulness of each in a field setting. The latter was accomplished by examining preserved museum specimens from collection sites in the Mediterranean and Atlantic and fresh specimens from Oregon.

MATERIALS AND METHODS

We compiled the literature on green crab species identification and created a list of characters that could potentially be useful for distinguishing *C. maenas* and *C. aestuarii* (Table 1). In addition, Dr. Toshio Furota kindly sent us his unpublished observations on the physical characteristics of *C. aestuarii* from Shinhama lagoon in Tokyo Bay Japan.

In order to evaluate the usefulness of each morphological feature in field identification, we obtained preserved specimens of similar size male *C. maenas* and *C. aestuarii* from the Smithsonian Museum of Natural History. Collection sites included Vlissingen, Netherlands, Chausey Islands in the English Channel, Rabat, Morocco, Tunis, Tunisia, and Marsala, Sicily (Fig. 2; Table 2). To supplement these samples, we collected live specimens from Yaquina Bay, Oregon using baited and pitfall traps. For each

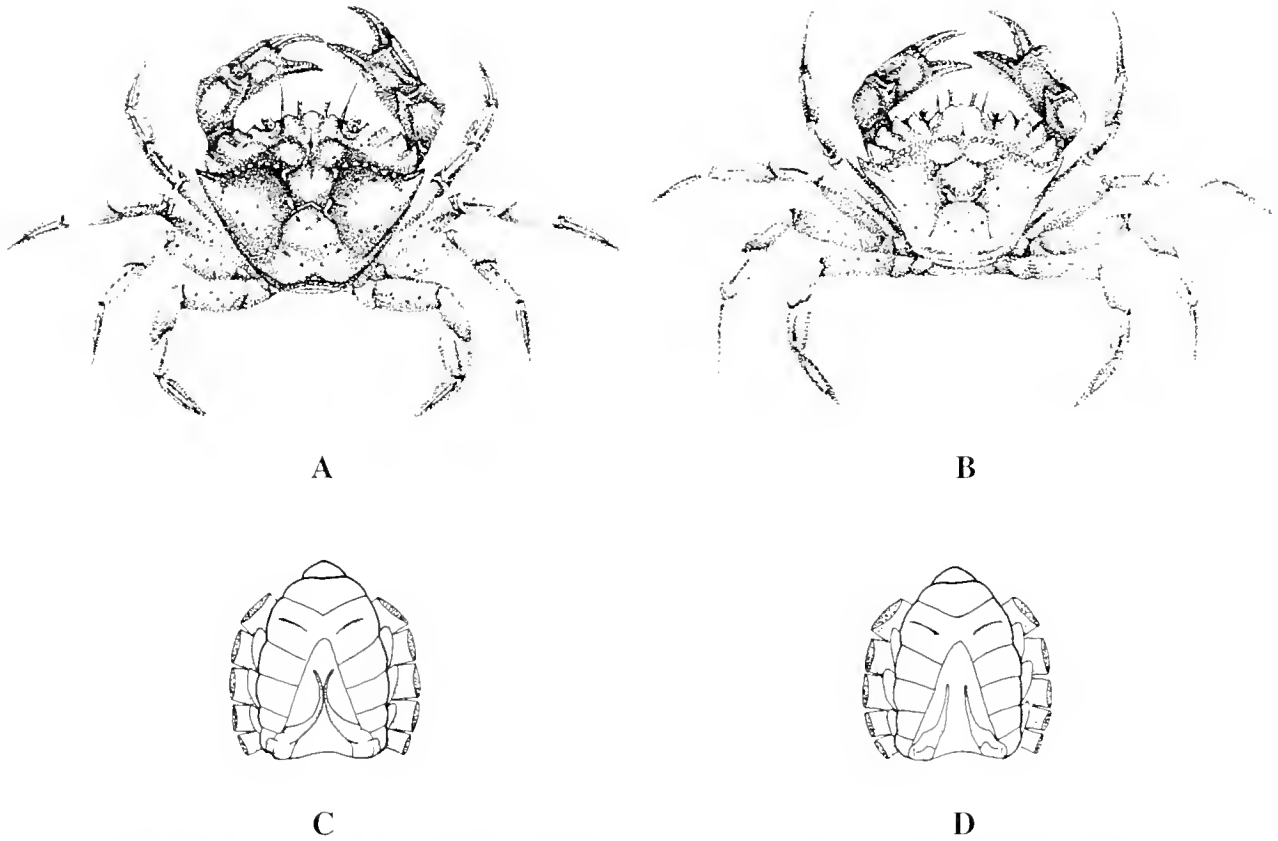

Figure 1. Distinguishing features of male specimens of *C. maenas* and *C. aestuarii*. Dorsal view of *C. maenas* (A) and *C. aestuarii* (B). Note that the carapace of *C. maenas* is wider than that of *C. aestuarii*. Typically, the frontal area of *C. maenas* is scalloped-shaped with distinct "bumps", while that of *C. aestuarii* is flatter and protrudes. The posterior-lateral margin of the carapace in *C. maenas* is generally convex and the 5th anterio-lateral tooth appears to point forward. The posterior-lateral margin in *C. aestuarii* is concave, while the 5th tooth is more elevated and appears to point outward. The legs of *C. maenas* appear shorter and thicker than those of *C. aestuarii*. Ventral view of breastplates with abdomen removed show the male copulatory appendages (pleopods). Note that the male pleopods are crescent-shaped in *C. maenas* (C) and straight and parallel in *C. aestuarii* (D). (Figure by Laura Hauck, reproduced with permission of Oregon Sea Grant.)

specimen we prepared a data sheet and noted which of the characteristics in Table 1 could be used as a diagnostic tool in a field setting. For a feature to be useful, it had to be visible to the naked eye (or through a hand lens) and measurable with vernier calipers.

RESULTS

Demeusy and Veillet (1953) and Demeusy (1953, 1958) draw attention to a suite of characters that distinguish green crabs from the Atlantic and Mediterranean coasts of France (Table 1). They obtained specimens from Sète in the Mediterranean and from La Manche (the English Channel) and Roscoff on the Atlantic (Fig. 2). They note that Mediterranean crabs are generally smaller, hairier, and possess a narrower and thicker carapace than Atlantic crabs. Almaça (1961) expands this comparative study by adding populations from the Spanish Mediterranean and the Spanish, Portuguese, and French Atlantic coasts (Fig. 2). While some of the characteristics were conserved across the larger sampling area, others, such as the distribution of hair on the carapace, frontal areas, and claws, carapace texture, and the number of segments on the antennae, were not (Table 1). Almaça (1972) observed carapace shape of *Carcinus* specimens from his former study areas as well as specimens from Australia and Maine and confirms that, in general, Atlantic specimens have wider and flatter carapaces than Mediterranean ones. He quantified these features as the carapace width (CW) to carapace length (CL) ratio (CW/CL) and the CW to carapace depth (CD) ration (CW/CD). For example, CW/CL is defined as the distance between the tips of the 5th anterio-lateral teeth divided by the distance between the central lobe of the frontal area and the posterior border of the carapace. CW/CL in *C. maenas* (≥20 mm) varied from 1.27 to 1.35, while that in *C. aestuarii* varied from 1.24 to 1.27. The CW/CD for males ≥20 mm was 2.27 to 2.57 for *C. maenas* and 2.25 to 2.31 for *C. aestuarii* (Table 3).

Zariquiey Alvarez (1968) provides a key for distinguishing the two species and includes photos of carapace shapes and male pleopods and a table of CW/CL for various size classes of both species and sexes (Table 4). The shape of the male pleopods is very diagnostic in separating adult Mediterranean from the Atlantic specimens (Fig. 1; Tables 1 and 2). When the abdomen of a male crab is lifted, the copulatory appendages, formed from the first two pairs of pleopods, become visible. The pleopods of *C. maenas* curve outward in a crescent shape with the centers of the crescents touching each other. Those of *C. aestuarii* form straight, parallel lines and do not touch. While size and sex appears to affect relative CW, the difference in the CW/CL between the species (for crabs ≥20 mm) is significant: 1.30 to 1.35 for *C. maenas* and 1.23 to 1.27 for *C. aestuarii* (Table 4). Rice and Ingle (1975) confirm

TABLE 1.

Morphological features useful for distinguishing *C. maenas* and *C. aestuarii* in the field. 1, Demeusey and Veillet 1953; 2, Demeusy 1953; 3, Demeusy 1958; 4, Almaça 1961; 5, Zariquiey Alvarez 1968; 6, Almaça 1972; 7, Rice and Ingle 1975; 8, Rasmussen 1973; 9, Noël 1992; 10, Furota 1999; 11, G. Jensen and C. Hieb, unpublished data; 12, Clark et al. 2001; 13, this study.

Feature	*C. maenas*	*C. aestuarii*	Comments
Native Distrubution (4,5)	Western Baltic, North Sea, Atlantic from Norway and Iceland to Morocco and Mauritania	Mediterranean, Black Sea, Asow Sea	Yes, for specimens within native range, (some mixing near Strait of Gibraltar)
Male pleopods (copulatory appendages) (4, 5, 9)	Crescent-shaped, touch at the bend	Parallel and straight, don't touch	Yes, Figure 1 (Fig. 115 in 5)
Posterior (5th) pair of teeth of anterio-lateral region of carapace (3, 5, 7, 13)	Appear to point forward	Appear to slant outward, tooth and central rib are elevated	Mostly, Figure 1, Teeth of some Atlantic specimens also slants outward (4)
Posterior-lateral margin of carapace (13)	Straight or convex	Concave	Mostly, Figure 1, But margin was concave in Atlantic specimen from Rabat, Morocco
Carapace width to length ratio (CW/CL) for crabs ≥20 mm (5, 6, 7, 9, 12, 13)	Wider carapace 1.29–1.36	Narrower carapace 1.22–1.27	Yes, Figure 1, Tables 2 and 3
Carapace width to depth ratio (CW/CD) (6, 12)	Thinner carapace 2.32–2.50	Deeper carapace 2.19–2.26	Shows great promise as a discriminator
Appearance of dorsal surface of carapace (1, 3)	Smooth and uniform	Cardiac, hepatic and brachial regions divided by deep furrows; rough with hairy projections	Not noticeable for preserved specimens (13)
Margin of frontal area between the eyes) (3)	Few or no bristles	Three size classes of bristles line the border of the frontal area	No (4, 7) Not noticeable for preserved specimens (13)
Shape of three lobes in frontal area (between the eye sockets) (5, 7, 13)	3 distinct "bumps", margin scalloped, frontal area does not protrude	Flatter, "bumps" not as distinct, frontal area protrudes beyond eyes	Yes, Figure 1 (Fig. 115 in 5, Plate 1 in 7) Toshio Furota, unpublished data
Segmentation of first antennae (3)	5–6 segments	4 segments	No, feature varies with size of animal (4)
Second antennae (3)	Fewer segments	Longer, more segments	No (4)
Orientation of teeth at the base of the second antennae (3)	On ventral side	On dorsal side	No (4)
Outer margin of claws (3)	Smooth	Hairy with bumps	No (13)
Tooth on inside of carpal (5)	Blunt	Sharp	No, (7, 13)
Presence of a fringe of long hair on the carpal ("wrist" segment) of claw (3)	No	Yes	No, some Atlantic specimens also possess this feature (4)
Length of legs	Shorter	Longer and more slender	Possibly, Figure 1, (Fig. 115 in 5) No (4)
Abdomen of same sized immature females (2)	Narrower	Wider (mature at a smaller size)	No, would be difficult to quantify
Carapace width of female in last prepuberty state (2)	22.5 mm	14 mm	No, great variability (d'Udekem d'Acoz, unpublished data)
Carapace width of females after puberty molt (2, estimate based on 35% growth increment with molt)	28–30 mm	16–19 mm	No, in Portugal ovigerous *female C. maenas* can be 15 mm and smaller (Kuris, unpublished data)
Maximum carapace width (5, 8, 10, 11)	>90 mm	65 mm	No, maximum size is not reached in many locations.

these species difference in CW/CL. The ratios for two female *C. maenas* from Plymouth, U.K. are 1.29 and 1.32, while that for a female *C. aestuarii* from Tunis, Tunisia is 1.25 (Table 3).

Clark et al. (2001) carried out the most statistically sound and geographically extensive study of carapace shape in *Carcinus*. They sampled 17 populations from the Atlantic, one from California, and eight from the Mediterranean and measured CW, CL, and CD for a total of 1,737 specimens. While the carapaces of the Atlantic and California populations were significantly wider and thinner than those of from the Mediterranean, there was also great variation between and within populations of a region. For example, the mean CW/CD for Atlantic populations varied from 1.27 to 1.32 and that for Mediterranean populations from 1.23 to 1.27. Standard deviations around the population means were over 0.02 (Table 3).

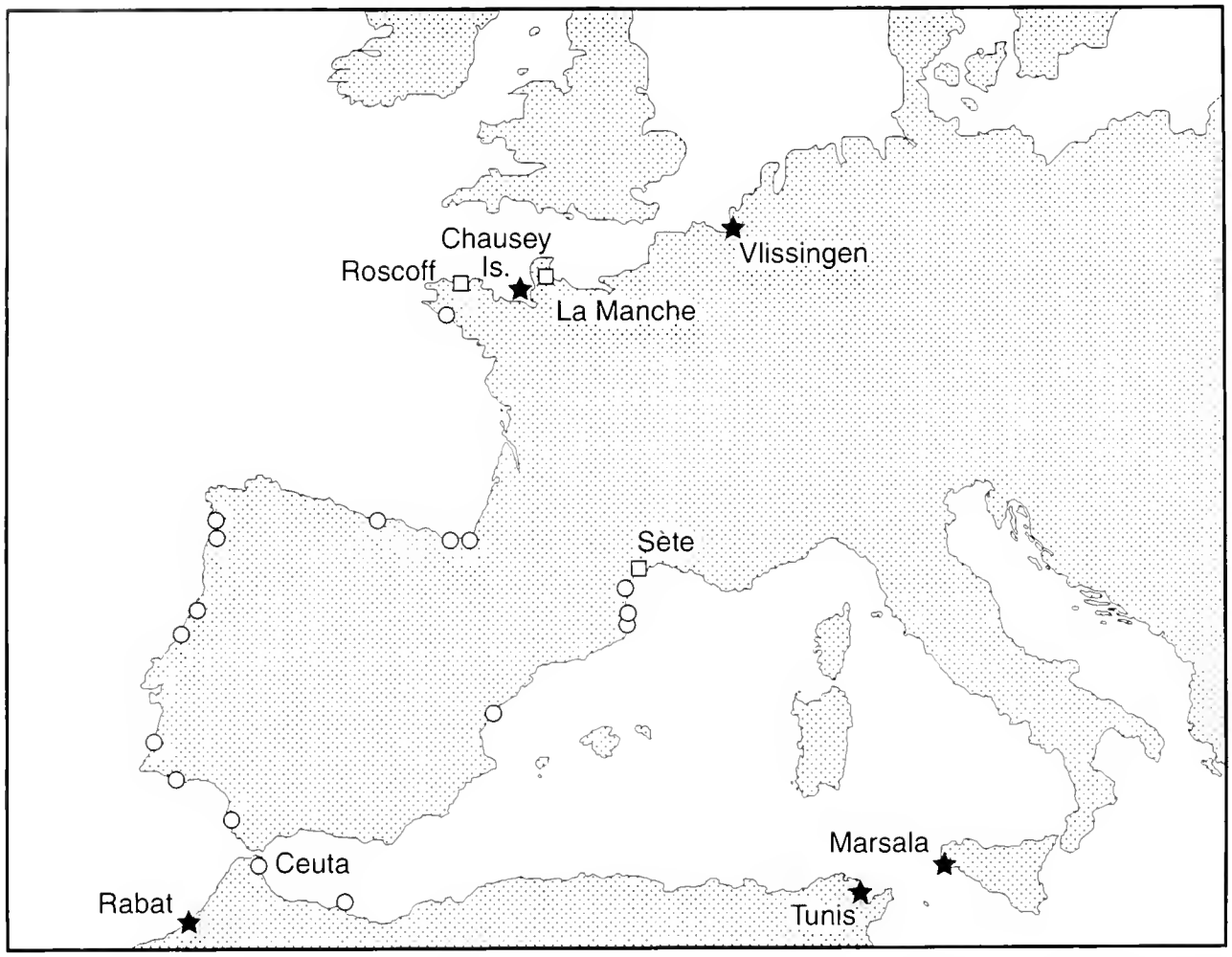

Figure 2. Location of collecting sites for Atlantic and Mediterranean populations of *Carcinus*. The study sites of Demeusy and Veillet (1953) and Demeusy (1953, 1958) are indicated by open squares and include Sète in the Mediterranean and La Manche (English Channel) and Rostcoff in the Atlantic. Almaça's (1960) study sites are indicated by open circles. Sites for museum specimens are indicated by dark stars and include Marsala and Tunis in the Mediterranean and Rabat, Chausey Islands and Vlissingen in the Atlantic.

The authors point out that it may not always be possible to correctly assign any one specimen to a species. They found less overlap in the mean CW/CD ratio: Atlantic populations varied from 2.32 to 2.50 and Mediterranean populations from 2.19 to 2.26 (Table 3).

Our study of a limited number of museum specimens from Europe and North Africa and fresh specimens from Oregon supports many of the previous findings. The shape of the male pleopods appears to be a good diagnostic field characteristic for separating adult males into the Atlantic and Mediterranean forms (Fig. 1; Table 2). The pleopods of *C. maenas* curve outward in a crescent shape with the centers of the crescents touching each other. Those of *C. aestuarii* form straight parallel lines and do not touch. All Oregon specimens clearly display the *C. maenas* pattern (Table 2). Another diagnostic feature is CW/CL. For our limited sample, we found that if the ratio is $\geq$1.29, then the specimen is *C. maenas*; if it is between 1.25 and 1.29, it is *C. aestuarii* (Table 2). Unfortunately, we failed to measure CW/CD ratio before returning our museum specimens.

After reviewing a previous version of this manuscript, Toshio Furota kindly supplied us with Table 5. This data set of carapace dimensions of *C. aestuarii* was collected by him and his coworkers while they were studying the life history of this species in Tokyo Bay (Furota et al. 1999). Furota states: "Results in morphological characteristics, including male pleopods and frontal area, clearly support that the samples are *C. aestuarii*." Both females exhibit a CW/CL ratio of under 1.27 and thus would be classified as *C. aestuarii*. One of the males exhibits a CW/CL of 1.31. While this value is not typical for *C. aestuarii*, it is within the range observed by Clark et al. (2001) (Table 3). The CW/CD ratios of 2.33 to 2.45 for three of the males, however, is unusually high for *C. aestuarii*. Almaça (1972) and Clark et al. (2001) found that CW/CD ratios of over 2.32 are characteristic of *C. maenas* (Table 3). The reasons for this discrepancy are unknown. Since CD is more difficult to standardize than CW and CL, Furota may have measured the crabs in a different part of the body than Clark et al. (2001). If, however, the difference remains after a larger sample size of crabs is examined using standard criteria, then a hybrid population may be present in Tokyo Bay.

We found that the shape of the lobes in the frontal area between the eye sockets tends to vary between the species. The three lobes of *C. maenas* possess distinct "bumps" and form a scalloped mar-

TABLE 2.

Morphological features of European and North African museum specimens and live crabs collected from Yaquina Bay, Oregon. CW/CW = carapace width divided by carapace length. An asterisk indicates that frontal area for the Moroccan specimen was difficult to categorize.

Collection Site	Smithsonian Catalogue No.	Carapace Width (mm)	CW/CL	Male Pleopod Orientation	Frontal Area	Species
Marsala, Sicily	205783	35.77	1.253	Straight	Protrudes	*C. aestuarii*
		27.54	1.263	Straight	Protrudes	*C. aestuarii*
Tunis, Tunisia	257965	42.55	1.279	Straight	Protrudes	*C. aestuarii*
Tunisia	257959	50.11	1.286	Straight	Protrudes	*C. aestuarii*
		47.85	1.263	Straight	Protrudes	*C. aestuarii*
South of Rabat, Morocco	258380	51.60	1.306	Curved	*	*C. maenas*
Chausey Islands, English Channel	283069	36.08	1.304	Curved	Scalloped	*C. maenas*
Vlissingen, Netherlands	119408	28.93	1.317	Curved	Scalloped	*C. maenas*
Yaquina Bay, Oregon		32.57	1.372	Curved	Scalloped	*C. maenas*
		35.66	1.331	Curved	Scalloped	*C. maenas*
		40.32	1.370	Curved	Scalloped	*C. maenas*
		44.53	1.350	Curved	Scalloped	*C. maenas*
		45.94	1.382	Curved	Scalloped	*C. maenas*
		75.17	1.348	Curved	Scalloped	*C. maenas*

gin between the eyes. The lobes of *C. aestuarii* lack distinct "bumps" and do not form a scalloped margin. The frontal area in *C. aestuarii* is flat and protrudes beyond the eyes (Fig. 1). Our specimen from Morocco was the only one that we found difficult to characterize. The three lobes did not have distinct bumps nor did the frontal area protrude beyond the eyes. The frontal areas of the introduced green crabs in Tokyo Bay are flat and protruding, supporting the view that the specimens are *C. aestuarii* (Table 5).

While male pleopods, carapace ratios, and the shape of the frontal area are good field characteristics for discriminating the two *Carcinus* species, other features can provide supportive evidence (Table 1). The fifth anterio-lateral teeth in *C. aestuarii* possess a higher central rib and appear more elevated than in *C. maenas*. The posterior-lateral margin of the carapace leading up to the 5th tooth tends to vary between the species. Typically, the margin is convex or straight in *C. maenas* and concave in *C. aestuarii* (Fig. 1). Thus, the fifth teeth appear to point forward in *C. maenas* and outward in *C. aestuarii*. While these features generally hold true, there can be exceptions. Our specimen from Rabat exhibits the typical concave pattern of *C. aestuarii*, but the straight pleopods of *C. maenas*.

TABLE 3.

Summary of carapace ratios of *C. maenas* and *C. aestuarii* observed by various researchers. Asterisks draws attention to the unusually high ratios for *C. aestuarii* from Tokyo Bay.

	C. maenas		*C. aestuarii*	
Study	CW/CL	CW/CD	CW/CL	CW/CD
Zariquiey Alvarez 1968	1.30–1.35		1.23–1.27	
Almaça 1972	1.27–1.35	2.27–2.57	1.24–1.27	2.25–2.31
Rice and Ingle 1975	1.29–1.32		1.25	
Clark et al. 2001 (range of population means)	1.27–1.32	2.32–2.50	1.23–1.27	2.19–2.26
Clark et al. 2001 (range of standard deviations of population means)	1.25–1.36	2.22–2.60	1.21–1.29	2.12–2.32
Furota unpublished data			1.25–1.31*	2.13–2.45*
This study	1.30–1.38		1.25–1.29	

The walking legs appear longer and more slender in *C. aestuarii* than in *C. maenas* (Fig. 1). While the carapace drawings are based on a composite of two specimens per species, great care was taken to correctly depict the relative proportions of the lengths and widths of all the limb segments. We feel that further investigation into a leg length to CL index might yield an additional diagnostic feature.

The last three distinguishing characteristics are related to life history features and are not useful in identifying crabs in the field. Sexual maturity in female *Carcinus* can be easily determined by simply attempting to lift the abdomen with a probe. If the abdomen is locked to the thorax, the female is immature, but if it lifts, she is mature. Males will mate with females prior to their puberty molt. Demeusy (1953) found that the smallest mated female *C. maenas* from the Atlantic coast of France was 22.5 mm in CW, while the smallest *C. aestuarii* from Sète in the Mediterranean was 14 mm. After molting, their CWs would be 28 to 30 and 16 to 19 mm, respectively (Table 1). Sexual maturity in *C. maenas* from Northern Europe, Maine, and Oregon typically occurs at a carapace width of 25 to 35 mm (Berrill 1982, Mohamedeen & Hartnoll 1989, d'Udekem d'Acoz 1993, Yamada 2001). Great variation in this feature, however, exists with females maturing at a smaller size in the fall than in the spring (d'Udekem d'Acoz 1993). In

TABLE 4.

Mean carapace width to length ratios (CW/CL) for male and female *C. maenas* and *C. aestuarii* of various size categories. Numbers in brackets give sample size. Data taken from Zariquiey Alvarez 1968.

Carapace width	Male *C. maenas*	Female *C. maenas*	Male *C. aestuarii*	Female *C. aestuarii*
0–10 mm	1.200 (1)		1.187 (5)	1.176 (5)
10–20 mm	1.272 (1)		1.224 (2)	1.281 (7)
20–30 mm	1.354 (2)	1.317 (5)	1.252 (11)	1.252 (9)
30–40 mm	1.323 (10)	1.304 (2)	1.266 (16)	1.259 (12)
40–50 mm	1.348 (9)	1.307 (1)	1.247 (7)	1.259 (1)
50–60 mm	1.327 (2)	1.298 (1)	1.264 (1)	1.227 (1)
Mean (CW > 20 mm)	1.336	1.305	1.256	1.249
Range (CW > 20 mm)	1.323–1.354	1.298–1.317	1.247–1.266	1.227–1.259

TABLE 5.

Carapace dimensions and characteristics of male pleopods and frontal area of green crabs collected from Shinhama lagoon in Tokyo Bay on May 5, 1999. This data set was provided by Toshio Furota. An asterisk indicates an unusually high ratio for *C. aestuarii* when compared with Mediterranean populations studied by Clark et al. (2001).

Sex	Carapace Width (mm)	Carapace Length (mm)	Carapace Depth (mm)	CW/CL	CW/CD	Male Pleopod Orientation	Frontal Area	Species
Male	51.7	39.5	22.1	1.31*	2.34*	Straight	Protrudes	*C. aestuarii*
	54.5	24.3	24.3	1.28	2.24	Straight	Protrudes	*C. aestuarii*
	53.6	42.3	23.9	1.27	2.24	Straight	Protrudes	*C. aestuarii*
	49.0	39.0	23.0	1.26	2.13	Straight	Protrudes	*C. aestuarii*
	46.5	36.3	19.0	1.28	2.45*	Straight	Protrudes	*C. aestuarii*
	39.4	31.6	16.9	1.25	2.33*	Straight	Protrudes	*C. aestuarii*
Female	29.9	23.9		1.25			Protrudes	*C. aestuarii*
	36.3	29.2		1.24			Protrudes	*C. aestuarii*

Belgium, the smallest mature female was only 22.1 mm, while the largest, in its pre-puberty molt, was 38.8 mm (d'Udekem d'Acoz, unpubl. data). In Mil Fontes, Portugal, *C. maenas* females 15 mm and smaller have been observed to carry eggs (Kuris, pers. comm.). Mori et al. 1990 report that size of sexual maturity for *C. aestuarii* from Sardinia occurs around 29 mm. Thus, size at sexual maturity of the female cannot be used to distinguish the two species of *Carcinus*. Maximum size attained by *C. maenas* is over 90 mm and 65 mm for *C. aestuarii* (Table 1). Since maximum size is not reached in many locations, this feature would be of limited usefulness in distinguishing the species.

DISCUSSION

While *Carcinus* populations exhibit variation from site to site, the greatest discontinuity in morphological and genetic features occurs near the Strait of Gibraltar. Morphological and genetic studies support the view that the Atlantic *C. maenas* and the Mediterranean *C. aestuarii* diverged from a common ancestor, but may still be subspecies rather than true species (Almaça 1961, Bulhnheim & Bahns 1996, Clark et al. 2001). The Strait of Gibraltar with its high sill prevents water masses of the Atlantic and Mediterranean from freely intermixing, thus creating two basins with distinct water chemistry. Some surface water, however, does flow from the Atlantic into the Mediterranean and some deeper, high-saline water does flow from the Mediterranean into the Atlantic (Hopkins 1985). Thus, the Strait of Gibraltar acts only as a partial barrier to larval and gene exchange (Almaça 1989, d'Udekem d'Acoz 1999). For example, Almaça (1961) reports *C. aestuarii* morphs from the Atlantic Canary Islands and *C. maenas* morphs from Ceuta, just inside the Mediterranean (Fig. 2). Hybridization may explain why our specimen from Rabat, Morocco exhibited features of both species. Furthermore, evidence is accumulating that a hybrid zone may exist on the southeastern coast of the Iberian Peninsula. Further studies are needed to clarify the extent of any gene exchange between the two forms near this region of contact.

From studying the literature and from examining specimens from Europe, North Africa, and Oregon, we conclude that in most cases, adult *C. maenas* and *C. aestuarii* can be distinguished in the field by the shape of the male pleopods, the frontal area, and the CW/CL. The pleopods of *C. maenas* curve outward in a crescent shape with the centers of the crescents touching each other. Those of *C. aestuarii* form straight, parallel lines and do not touch. The Oregon specimens exhibit *C. maenas*-shaped male pleopods. While male pleopods are diagnostic in species identification, one needs to be aware of some special circumstances when they are not. For example, when male *C. aestuarii* are parasitized by the castrating barnacle, *Sacculina carcini*, the pleopods are often curved (Noël, pers. comm.). Almaça (1961) cautions that pleopods may not be diagnostic in immature males before the pleopods harden. He states: "The thin pleopods of immature Atlantic specimens can be as straight as those from the Mediterranean."

We found that the shape of the three lobes in the frontal area between the eye sockets tends to vary between the species. The three lobes of *C. maenas* possess distinct "bumps" with a scalloped margin between the eyes. The lobes of *C. aestuarii* are flatter, less distinct, and protrude beyond the eyes. The frontal areas of the introduced green crabs in Tokyo Bay are flat and protruding, confirming that the specimens are *C. aestuarii* (Furota, pers. comm.). All of our specimens, with the exception for the one from Morocco, were correctly categorized to species using this feature. This observation supports the view that hybridization may occur near the Strait of Gibraltar.

Another distinguishing feature is that the carapace of adult *C. maenas* is wider than that of *C. aestuarii*. This feature can be quantified as the CW/CL ratio. Zariquiey Alvarez (1968) finds that for each size and sex category, *C. maenas* has a larger CW/CL ratio than *C. aestuarii* and that *male C. maenas* typically have larger CW/CL ratios than females. This sex difference in adult *C. maenas* was also observed by Shen (1935) who reports a CW/CL of 1.31 for males and 1.29 for females. Studies by Almaça (1972), Rice and Ingle (1975), Clark et al. (2001), and this study confirm the observation that *C. maenas* has a larger CW/CL than *C. aestuarii* (Table 3). Clark et al. (2001) point out that because of natural variability within a population, it is not always possible to accurately identify any one specimen. However, if an adult *Carcinus* specimen exhibits a CW/CL of over 1.29, it most likely is *C. maenas*; if it exhibits a CW/CL of under 1.27, it most likely is *C. aestuarii*. Two of our specimens from Tunis with CW/CL just under 1.29 would have presented a classification problem had it not been for their distinct straight pleopods and flat, protruding frontal areas. Female *Carcinus* with borderline CW/CL ratios, however, would be difficult to classify to species.

The great variability observed in the CW/CL of the introduced *C. aestuarii* in Tokyo Bay could support an hypothesis proposed by Geller et al. (1997) that this population is composed of hybrids. Molecular genetic analysis revealed mitochondrial haplotypes

characteristic of both species. The authors suggest either that the population originated from the hybrid zone near the Strait of Gibraltar or that more than one species has been introduced into Tokyo Bay. A subsequent analysis using microsatellite DNA did not support the multiple-invasion hypothesis for this population (Bagley & Geller 2000). Clearly, more research is needed to clarify the origin of this introduced population of *Carcinus*.

While the two sibling species of *Carcinus* may look alike, it is possible to distinguish them in the field using the above morphological criteria. By incorporating the three defining characteristics, those of the shape of the male pleopods, the shape of the frontal area between the eye sockets, and the CW/CL ratio, successful field differentiation between these two species can be achieved in most cases. Other features, such as the orientation of the 5th antero-lateral spine and the curvature of the posterior-lateral carapace margin are not diagnostic, but can provide supporting evidence for species identification.

We believe that two additional features hold great promise as good species discriminators. We propose that the CW/CD ratio and the relationship of the length and width of the walking legs to CL be investigated. Furthermore, we suggest that greater discrimination between the species could be achieved by taking the sexual difference in the carapace shape into account.

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REDUCING PREDATION ON MANILA CLAMS BY NONINDIGENOUS EUROPEAN GREEN CRABS

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ABSTRACT The introduced European green crab (*Carcinus maenas*) poses a potential risk for commercial production of Manila clams (*Venerupis philippinarum*), a growing fishery in western North America. We investigated methods for modifying commercial production of Manila clams in order to reduce losses to green crab predation. In both 1997 and 1998, the timing of the outplant of seed clams was varied such that one portion of the total clam production was outplanted early in the year (March) and another portion outplanted later in the year (August/September). In July and August 2000, we examined these bags outplanted in 1997 and 1998 and quantified the clam mortality and the abundance of crabs including green crabs. We tested the effects of both year of transfer (1997 vs. 1998) and the timing of transfer (early season vs. late season) on clam and crab abundance and found significantly ($p < 0.05$) fewer green crabs and less predation on Manila clams in bags that were outplanted in the late season. Year of outplant also influenced clam survival and green crab abundance, such that delayed outplant significantly ($p < 0.05$) increased clam survival only when green crab abundances were high (1997). Our data suggest that the increased Manila clam survival in delayed outplant bags was likely due to reduced green crab recruitment and consequently lower green crab predation. We conducted additional experiments on relative predation rates by green crabs on Manila clams. We found that green crab predation is strongly size dependent, and that while juvenile green crabs were not effective predators of market size Manila clams (>36 mm in width), these clams were easily consumed by adult green crabs (>50 mm carapace width). We conclude that in years of high green crab abundance, delayed outplant is an effective means of reducing losses of commercially produced Manila clams.

KEY WORDS: Manila clam, *Venerupis philippinarum*, European green crab, *Carcinus maenas*, predation, recruitment, nonindigenous

INTRODUCTION

More than 400 non-native species have become established in the coastal waters of the U.S., and many have resulted in substantial economic and ecological impacts (Ruiz et al. 1997, Cohen & Carlton 1998, Ruiz et al. 2000). In western North America, the recently introduced European green crab (*Carcinus maenas*, Linnaeus, 1758) now poses a potential threat to the commercial production of Manila clams (*Venerupis (Ruditapes) philippinarum*, A. Adams and Reeve, 1850) (see Carlton 1992 and Coan et al. 2000 regarding subspecies) and other invertebrate fisheries in western North America (Lafferty & Kuris 1996, Jamieson et al. 1998). Like green crabs, Manila clams are also non-native, having been accidentally introduced with Pacific oysters (*Crassostrea gigas*, Thunberg, 1795) in San Francisco Bay in the 1940s (Cohen & Carlton 1995). Manila clams now support a growing commercial fishery with landing revenues of approximately $10.5 million in 1999 for Washington (Pacific States Marine Fisheries Commission 1999) and increasing production in Oregon, California and British Columbia, Canada. European green crabs have the potential to become an important source of mortality for Manila clam producers throughout most of this range, where they have been reported from Morro Bay, CA to Vancouver Island, British Columbia since 1998, and have established populations between Monterey Bay, CA and Gray's Harbor, WA (Grosholz et al. 2000, G. S. Jamieson, unpub. data). In central California, where they first became established in San Francisco Bay in 1989, green crab predation has already resulted in losses of nearly 40% of the annual production of 5,500 kg for one producer in 1996 (John Finger, Hog Island Oyster Co., Marshall, CA, pers. comm.).

European green crabs are widely acknowledged as a pest species in other areas including Australia (Thresher 1997), South Africa (LeRoux et al. 1990, Griffiths et al. 1992), and the northeastern U. S., where they likely contributed to the demise of the soft-shell clam (*Mya arenaria* Linnaeus, 1758) fishery in New England in the early 1900s (Glude 1955, MacPhail et al. 1955, Ropes 1968). In its native range along the North Atlantic coast of Europe, green crabs are prodigious consumers of invertebrates in general, and bivalves in particular (Reise 1977, Scherer & Reise 1981, Jensen & Jensen 1985, Sanchez-Salazar et al. 1987, Rafaelli et al. 1989) and are one of the primary predators of Manila clams (Cigarría & Fernández 1998, Cigarría & Fernández 2000).

The method of commercial production is likely to influence rates of green crab predation on Manila clams. Several culture methods are used in this fishery depending on available space, substrate type, and local predators, include bag culture, and direct outplanting (Spencer et al. 1992). During the two to four years required to grow Manila clams to market size, they can be vulnerable to various threats including crabs, waterfowl, fouling organisms, and parasites (Laing 1993, Paillard & Maes 1994, Smith & Langdon 1998). After the losses to green crabs experienced by central California growers in 1996, there was a need to modify the bag culture methods commonly used for Manila clam production in central California, in order to reduce green crab predation.

The specific goal of this study was to investigate the effectiveness of delaying the timing of outplant of Manila clams for reducing predation by green crabs. Typically, hatchery reared seed clams are initially placed in small mesh "seed bags," then, after approximately one year, are transferred from the seed bags to larger mesh "growout bags." The original date of outplant into seed bags largely determines the timing of the transfer of clams from seed bags to growout bags, so that both outplant and transfer happen either early in the season (winter/spring) or later in the year (summer/fall) approximately a year apart. After transfer, clams remained in growout bags for typically two more years without any further management. We hypothesized that the timing of the

dates of outplant and transfer (hereafter referred to as outplant date) from early in the year (March) to later in the year (August/September) would reduce the number of green crabs that recruited into the Manila clam bags as megalopae or early instar juveniles. Since predation is cumulative during the time Manila clams spend in both seed and growout bags, our assumption was that reducing green crab recruitment into clam bags would reduce the abundance of predatory adult green crabs, thus resulting in higher survival of Manila clams at the time of harvest.

MATERIALS AND METHODS

Manila clams used in these experiments were grown in commercial culture by the Hog Island Oyster Company (HIOC), Marshall, CA, USA. The site for the delayed outplant experiment was Tom's Point in Tomales Bay (Marin Co.), CA (38°13′02″N, 123°56′49″W). The site has a muddy sand substratum and an annual temperature range of 10–20°C and a salinity range of 20–33 ppt. Salinity remains high during drier months from late spring through early fall (Cole et al. 1990 for additional site information on Tomales Bay).

To investigate size dependent predation of Manila clams by green crabs, we conducted an additional short-term experiment on the marine reserve of the Bodega Marine Laboratory, Bodega Bay (Sonoma Co.), CA (38°20′00″N, 123°2′30″W). This site has sandy-muddy sand substratum with a temperature range similar to Tomales Bay, but with salinity generally near full seawater. Additional site information can be found in Grosholz et al. 2000.

Delayed Outplant Experiment

The experiment consisted of two experimental treatments: the first treatment involved manipulating the timing of outplant (and transfer) and the second treatment was the year that the clams were first outplanted (either 1997 or 1998). Early outplant occurred in March (1997 and 1998) and late outplant occurred in September (1997) and August (1998). This resulted in bags being in the field exposed to crab predation for approximately the following number of days: 1,215 days (March 1997), 1,035 (September 1997), 850 (March 1998), 700 (August 1998). Outplanted seed clams (approximately 6 mm width) were held in seed bags (3 mm mesh bags, 1 m L × 0.6 m W × 0.3 m H) that were placed in rows on the substrate at a tidal height between approximately +0.3 m and +1.0 m. After approximately one year, clams (approximately 12 mm width) were transferred from seed bags to growout bags (6 mm mesh, 1 m L × 0.6 m W × 0.3 m H) and typically remained in growout bags for an additional two years until they reached commercial size. During this transfer from seed bags to growout bags, larger crabs and excess shell debris were removed, although smaller shore crabs such as *Hemigrapsus oregonensis* (Dana, 1851) were frequently missed and not removed during transfer.

Between July 2000 and September 2000, we examined bags that had been outplanted both early and late in 1997 and 1998. For each of the four different outplant periods, March 1997, September 1997, March 1998, and August 1998, we examined at least 20 Manila clam bags. For each bag, we recorded the number, size (carapace width to nearest mm), species, and gender of all crabs including European green crabs (*Carcinus maenas*), grapsid shore crabs including *Hemigrapsus oregonensis* and *Pachygrapus crassipes* (Randall, 1839), and cancrid crabs *Cancer productus* (Randall, 1839), *Cancer magister* (Dana, 1852), *Cancer antennarius* (Stimson, 1856). Also, we recorded the number of crushed clams by counting intact hinges and measuring a subset of the remaining live clams at the widest point on the shell to nearest mm. Shells crushed by green crabs were generally identified by characteristic shell damage at the growing edge of the shell. Since clams outplanted in 1997 were already market size, we examined these at the HIOC facility as they were being harvested for sale. However, since clams outplanted in 1998 were slightly smaller and not ready for harvest, we examined these in the field using the same data collection methods, and returned them to the field.

To address the possibility that differences in the relationship between shell thickness and shell width for clams outplanted early vs. late might contribute to differences in predation rates, we measured the shell width along the anterior-posterior axis and shell thickness at the posterior margin and at the pallial sinus for 100 intact shells from March 1998 and August 1998.

Size Dependent Predation Experiment

We conducted a second experiment in July 1999 to examine how the relationship between green crab size and Manila clam size influence predation rates under commercial culture conditions. Using commercial growout bags from HIOC, we placed 15 clams chosen from one of three size class treatments (<23 mm, 25–33 mm, or >36 mm) in commercial clam bags with one green crab chosen from one of three size class treatments (30–45 mm, 50–65 mm, or >70 mm). All experimental crabs for the two largest size classes were intermolt males, while for the smallest size class, because of the limited availability of intermolt males in this size range, we used half males and half females split evenly among clam size treatments. We used a fully factorial design with all treatment combinations yielding nine treatments (3 clam × 3 crab treatments) with each treatment replicated five times for a total of 45 experimental units (=clam bags).

We placed the 45 growout bags with experimental clams and crabs in five blocks placed at approximately 0.2 MLLW in Bodega Harbor anchored to the substrate following standard commercial outplant procedures. We anchored bags placed side by side and separated blocks by approximately 5 meters. After two weeks, we recovered all clam bags and counted and measured all clams and crabs.

Because there was no shell breakage or other physical evidence of crabs in the smallest size class preying on clams in the largest size class, we used this treatment as a conservative "control" treatment, for which we assigned mortality in this treatment as "non-predator mortality." We used this value for "non-predator" mortality (4%) to recalculate mortality for other treatments prior to analysis. Therefore, this correction of 4% reduces the between-treatment differences in mortality between bags with large, medium, and small crabs prior to statistical analysis. This resulted in more conservative between-treatment tests by making it more difficult to determine significant differences between treatment, thus reducing the probability of Type II error.

Statistical Analysis

For the outplant delay experiment, we conducted a two-factor analysis of variance (ANOVA) with year (1997 or 1998) crossed with outplant date (early or late) as main effects and the number of green crabs per bag and the number of preyed clams per bag as dependent variables. Separate post-hoc tests of treatment means were estimated with Tukey's studentized range test for each year.

To analyze the relationship between the number of green crabs per bag and the number of preyed clams across all outplant periods, we used linear regression with the numbers of green crabs and the numbers of preyed clams as paired variates for all outplant dates. We used linear regression to analyze the relationship between green crab abundance and several other variables including the number of preyed clams per bag, the number of *Hemigrapsus oregonensis* per bag, and the number of *Pachygrapsus crassipes* per bag. We also used linear regression to analyze the relationship between clamshell thicknesses on clam width for clams outplanted early vs. late in 1998. Lastly, to test for differences in size-specific shell thickness between treatments, we conducted independent linear regressions of shell thickness on shell width for both March and August 1998 clams (100 each) using ordinary least squares regression.

For the size-dependent predation experiment, we used a two-factor ANOVA with crab and clam size as main effects and clam survival as the dependent variable. As a limited test the effect of crab gender on clam mortality, we also analyzed data from this experiment in treatments with the smallest crab size class where we used equal numbers of male and female crabs. We used a t-test to compare differences in mortality for males vs. female crabs pooled across clam size treatments (n = 15).

The data for all ANOVAs were tested for homogeneity of variances and data were either square root transformed (counts), log-transformed (sizes), or arc-sin square root transformed (percentages) as needed to meet test assumptions. All statistical analyses were conducted with SAS 8.0 (Statistical Analysis Systems, Carey, NC).

RESULTS

Delayed Outplant Experiment

Overall green crabs were most abundant in bags outplanted in 1997 and were more abundant in bags outplanted early (March 1997) than late (September 1997) (Fig. 1). Both the outplant date ($F = 4.72$, $p < 0.05$) and year ($F = 16.3$, $p < 0.0001$) significantly affected the average number of green crabs per bag, and there was a significant interaction between outplant date and year ($F = 5.63$, $p < 0.05$). Post-hoc tests showed there was a strong affect of outplant date in 1997 with fewer crabs in clam bags on the later outplant date ($F = 6.8$, $p < 0.015$), but there was no significant effect of outplant date in 1998 ($F = 0.16$, $p > 0.60$) when overall green crab abundances for that year were lower (Fig. 1). Although the mean abundance of green crabs was low (mean 0.8 ± 0.66 crabs per bag in March 1997), only one green crab per bag may be necessary to produce large losses of clams (see later).

A strong treatment effect was evident with the percentage of crushed clams per clam bag being greatest in the March 1997 outplant group (mean 45.5% ± 21.4) (Fig. 2). Overall, there was a highly significant effect of both year ($F = 66.74$, $p < 0.0001$) and outplant date ($F = 31.08$, $p < 0.0001$) and there was a significant interaction as well ($F = 17.31$, $p < .0005$). Post-hoc tests also showed a strong affect of outplant date in 1997 ($p < 0.0001$), but not in 1998 ($p > 0.40$).

We also found a strong positive relationship between the number of green crabs per bag and the number of crushed clams, when data were pooled across outplant dates. Figure 3 shows that as the number of green crabs per bag increases, there was a significant increase in the number of preyed clams observed per bag ($n = 77$, $r^2 = 0.427$, $p < 0.001$). We also found that the abundance of *Hemigrapsus* was negatively associated with the abundance of green crabs. As Figure 4 shows, there was a significant negative relationship between the number of *Hemigrapsus* per bag and the number of green crabs per bag ($n = 81$, $r^2 = 0.20$, $p < 0.001$). Our analysis of the effects of year and outplant period on the number of *Hemigrapsus* showed a significant affect of year ($F = 7.39$, $p < 0.01$), no effect of outplant date ($F = 0.07$, $p > 0.70$), and a significant year by outplant date interaction ($F = 11.21$, $p < 0.005$).

The mean size of the clams in the growout bags varied as a function of outplant date (36.3 ± 4.9 mm March 1997, 34.1 ± 3.1 mm September 1997, 33.5 ± 2.9 mm March 1998, 32.4 ± 2.4 mm August 1998) (Fig. 5). Clams from early outplant date groups (March 1997 and March 1998) were significantly larger ($F =$

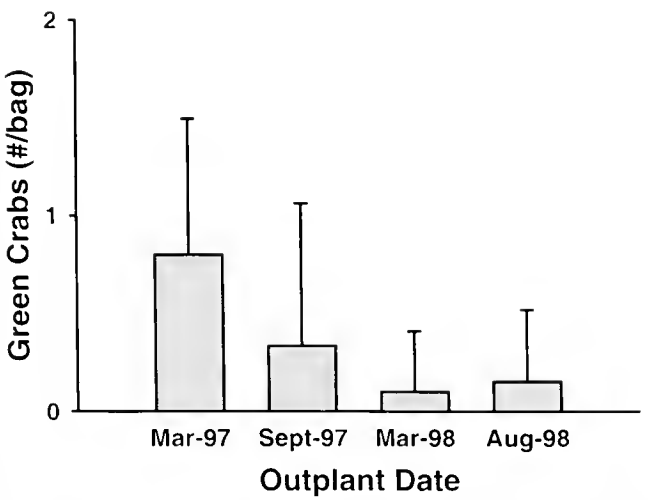

Figure 1. The number of green crabs observed per Manila clam bag for four outplant dates in outplant delay experiment (see text for total number of days in field). Bar heights represent the mean number of green crabs for 20 clam bags sampled from each outplant date. Error bars represent one standard deviation.

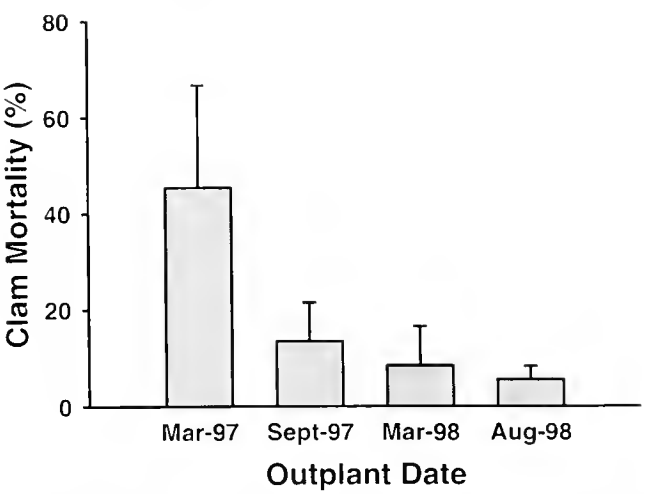

Figure 2. The percentage of crushed clams (=predation, see text) of total per Manila clam bag from four outplaot dates in outplant delay experiment. Bar heights represent the mean numher of crushed clams for 20 clam bags sampled from each outplant date. Error bars represent one standard deviation.

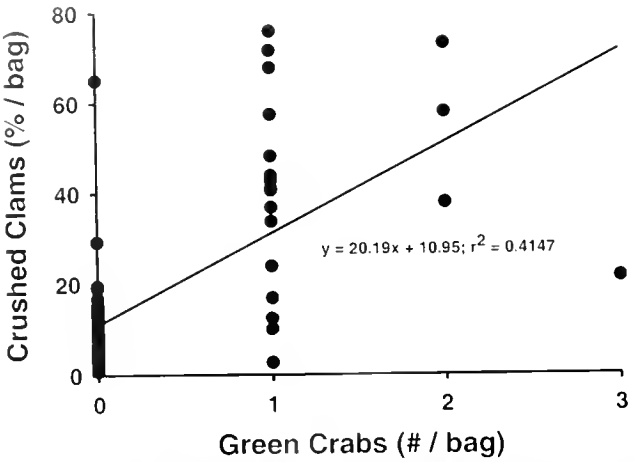

Figure 3. The number of crushed Manila clams versus the number of green crabs per Manila clam bag in the outplant delay experiment. Each point represents the total number of crabs and clams for each of twenty bags for all four outplant periods (March 1997, September 1997, March 1998, August 1998).

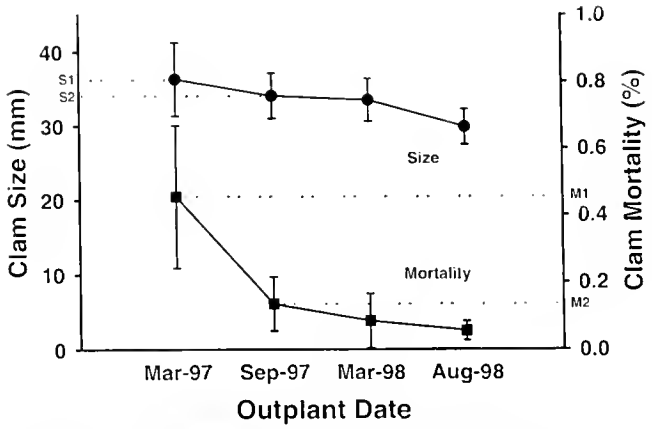

Figure 5. The size of Manila clams and the mortality measured in the delay outplant experiment. Each point represents the mean size of Manila clams (circles scaled on left axis) or mean percentage mortality of Manila clams (squares scaled on right axis) for each of twenty bags for all four outplant periods (March 1997, September 1997, March 1998, August 1998). The difference in mean clam size for March 1997 relative to September 1997 is indicated on the by S1-S2 on the left axis, and the difference in mean clam mortality for this same period is indicated by M1-M2 on the right axis.

15.3, $p < 0.001$) than clams from late outplant date groups (September 1997 and August 1998) and clams outplanted in 1997 were significantly larger (width) than clams outplanted in 1998 ($F = 27.8$, $p < 0.001$). These differences in mean size are the simple outcome of earlier initial outplant, thus more time to grow larger. These patterns of size variation, either as a function of outplant period or outplant year, indicate that our clam predation data are not simply the result of increased survival of larger clams, since the treatments with largest clams (March 1997) had the highest mortality.

These patterns in clam size resulting from different outplant times did not effect the relationship between clam size (width) and shell thickness. We found no significant differences in the relationship between shell thickness (in mm) measured at both the posterior edge, which was the portion of the shell typically crushed by crab predators, and shell width (in mm) measured as the maximum length along the anterior-posterior axis (Fig. 6). Neither slope was statistically different from zero ($p >> 0.10$). Using shell thickness measured at the pallial sinus gave the same answers. There was a slight trend towards longer shells having slightly thicker shells (although there were a small number of small, thick shells), but within the narrow size range of clams used in these experiments, this trend was not significant ($p > 0.60$ for March, $p > 0.20$ for August).

Lastly, green crab size and gender were similar between treatments (Table 1). There were with no significant differences ($p > 0.80$) among treatments in crab size and an increasing trend in crab sex ratio (male:female) with treatment date, although the low numbers of crabs in 1998 make these ratios a bit suspect.

Size Dependent Predation Experiment

The relative sizes of both green crabs and Manila clams influenced predation rates of Manila clams (Fig. 7). There was a sig-

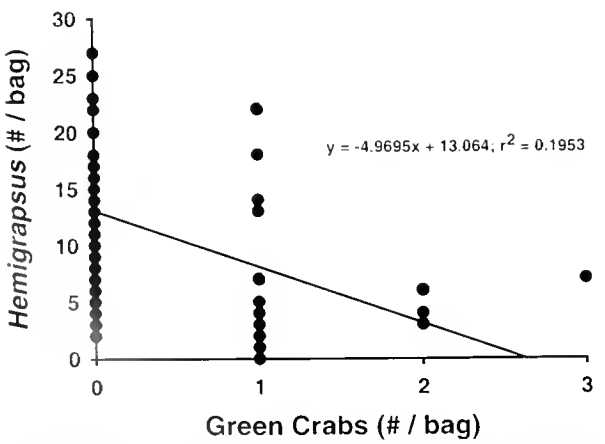

Figure 4. Number of green crabs observed per clam bag versus the number of native shore crabs (*Hemigrapsus oregonensis*) in outplants delay experiment. Each point represents the total number of crabs and clams for each of twenty bags sampled from all four outplant periods (March 1997, September 1997, March 1998, August 1998). Regression line is an ordinary least squares fit through all points with an unconstrained intercept.

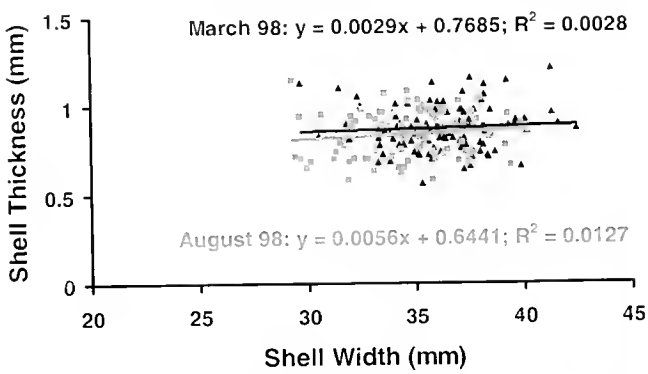

Figure 6. The relationship between shell thickness and shell width for Manila clams from two outplant dates (March 1998 and August 1998). Each point represents single measurements of thickness and width of a single shell from either March 1998 (black triangles) or August 1998 (gray squares). Regression lines for either March 1998 (black) or August 1998 (gray) are ordinary least squares fit through all points.

TABLE 1.

Abundance and size of male and female green crabs (*Carcinus maenas*) that recruited into Manila clam bags during the Delayed Outplant Experiment. For each outplant period (n = 20 bags), the mean and one standard deviation are shown for green crab abundance per bag (Mean # and S.D. #) and green crab carapace width (Mean CW and S.D. CW) for both males and females as well as male to female ration (M : F ratio).

	Mar-97	Sep-97	Mar-98	Aug-98
Males				
Mean #	0.30	0.14	0.05	0.10
S.D. #	0.47	0.36	0.00	0.31
Mean CW	54.17	59.33	36.00	41.00
S.D. CW	19.23	4.93	0.00	15.56
Females				
Mean #	0.50	0.19	0.05	0.05
S.D. #	0.61	0.51	0.00	0.00
Mean CW	57.10	56.00	70.00	48.00
S.D. CW	11.17	9.13	0.00	0.00
M : F Ratio	0.6	0.74	1	2

nificant effect of green crab size ($F = 12.6$, $p < 0.0001$) and Manila clam size ($F = 6.37$, $p < 0.005$) and no significant ($p > 0.50$) interaction. We found that both medium and large green crabs preyed upon substantial numbers of large Manila clams. However, small green crabs (30–45 mm) were not able to prey on the large Manila clams (>36 mm) (Fig. 5) and no crushed shells were seen in any replicates in this treatment. We found no significant effects of crab gender with similar survival of clams in bags with male (mean 84% ± 13) vs. female crabs (mean 86% ± 15) ($t = -0.28$, $df = 13$, $p > 0.70$) using bags with the smallest crab size class (to compare males or females) pooled across clam treatments.

DISCUSSION

The main result was the strong effect of outplant timing on the number of crushed clams and the number of green crabs in clam bags. Clams outplanted early (March 1997) had higher numbers of green crabs and experienced greater losses than clams outplanted later (August 1997) (Fig. 1). These data suggest that in growout bags outplanted in March 1997, adult green crabs were capable of consuming an average of 45% of Manila clams, and in the most extreme cases, more than 70% (Fig. 2).

The regression analysis showed a significant ($p < 0.001$) positive relationship between increasing numbers of green crabs in growout bags and increasing numbers of crushed clams (Fig. 3). However, this relationship explained only about 40% of the variation in clam mortality, due largely to bags with "zero" crabs and substantial numbers of crushed clams. It is possible that the number of green crabs in these bags was initially higher, but unknown mortality may have resulted in fewer crabs per bag. Because the results show modest numbers of green crabs per bag (on average less than 1 green crab per bag) even for clams outplanted in March 1997 (Table 1), this suggests that the high mortality in the early-outplanted bags was produced by small number of crabs. In any case, the substantial numbers of crushed clams in these experimental bags underscores the potential impact that green crabs can have on clams in commercial culture. Although other factors can also produce substantial clam mortality, we are unaware of any that would cause the conspicuous shell damage observed along the margin of the shell (typically posterior margin) inside clam bags other than crab predation.

Another result from our study is that the effect of the timing of outplant was much smaller and not significant ($p > 0.60$) in 1998 in comparison with 1997, when overall green crab abundance were much lower. This suggests that the impact of green crabs may be variable between years, and if green crab recruitment is poor, as in 1998, there may not be sufficient green crab predation to justify the delay in outplant (and transfer) given some cost to delayed outplant in terms of growth (see Fig. 5).

The decreased predation in clam bags outplanted later may be partly the result of less total time spent in the field. In Figure 1, there is a positive relationship between the number of days the bags were in the field and the number of crushed clams. Given that the March 1997 treatment had been in the field for three recruitment seasons and the March 1998 treatment had been in the field for only two, it might be expected that the March 1997 treatments might have more crabs or the crabs may have attained a larger size.

The delay in outplant timing had a stronger effect on reducing predation than reducing clam size. As shown in Figure 6, we found a 70% reduction in mortality between clams outplanted in March 1997 (mean 45.5% ± 21.3) vs. September 1997 (mean 13.5% ± 8.0) (M1-M2 in Fig. 6), but only a 6% reduction in size between clams outplanted in March 1997 (mean 36.3 mm ± 5.0) vs. September 1997 (mean 34.1 ± 3.1) (S1-S2 in Fig. 6). Thus, the delayed outplant resulted in a substantial increase in clam survival, and therefore, commercial production, with a relatively minor reduction in clam size.

This differential effect on the number of days in the field on mortality vs. size likely reflects the possibility that green crab recruitment may not be as continuous throughout the year as predation. The number of spring/early summer recruitment seasons that a bag may experience may largely determine the number of crabs per clam bag. Green crabs are able to enter the growout bags only while they are smaller than the mesh, such as megalopae and early instar juveniles (generally CW <10 mm, thus carapace height <6 mm). In central California, most recruitment of early instar green crabs occurs during the spring and early summer (Grosholz et al. 2000, Grosholz, unpubl. data) and by late summer, most

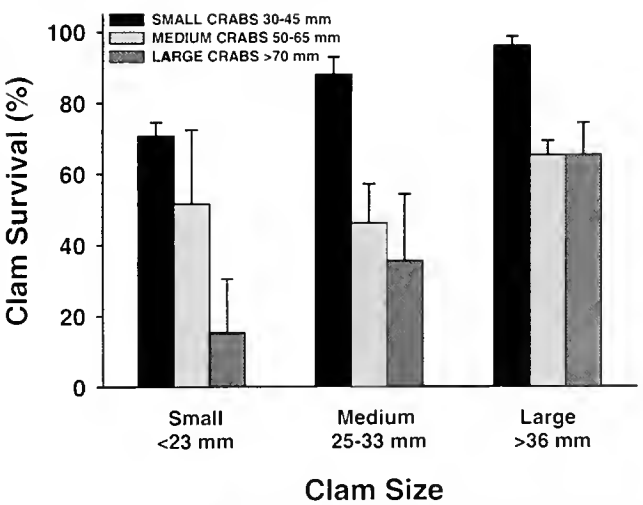

Figure 7. Percentage survival of Manila clams in the size dependent predation experiment. Bar heights represent the mean survival of clams for each of the nine treatment combinations (n = five bags per treatment). Error bars represent one standard error of the mean.

green crabs are too large to enter the growout bags. Therefore, clams bags outplanted in the fall would not generally be subject to recruitment by young-of-the-year green crabs, although they would be receive recruits in the following years.

The size dependent predation experiment provides additional support for green crabs being responsible for most of the clam mortality observed in the outplant delay experiment. Although green crabs in the smallest size class (30–45 mm CW) were not able to consume market-size clams (>36 mm), we found medium size crabs (50–65 mm CW) could consume Manila clams of any size in commercial production, so there was no size refuge from green crab predation (Fig. 7). Also, mortality of the largest clams was the same for both medium and large green crabs suggesting that green crabs in central California, which typically reach sizes exceeding 50 mm CW in their first year (Grosholz & Ruiz 1995, Yamada, in press), can consume the full range of clam sizes in commercial bags within a one year of recruitment. In addition, within the limited size range and small samples sizes involved in this experiment, we found no significant differences in predation levels between equivalently sized male and female green crabs ($p > 0.70$).

Although the outplant timing appears to have influenced clam mortality, many other characteristics of the crabs and clams in these experiments can influence the observed patterns of mortality. Previous work by Elner and Hughes (1978) Elner (1980, 1981), and Elner and Rafaelli (1980) have demonstrated that characteristics of crabs such as chelae size, sex, molt stage, etc. may interact with previous diet experience, temperature and other environmental factors to influence the rate and nature of predation. In the delayed outplant experiment, because we could not control the identity of the crabs in the clam bags (crabs recruited freely into bags), we were unable to control for differences among crabs. However, data for crab characteristics shown in Table 1 suggest that the differences in mortality among treatments were unlikely to have been influenced by differences in crab size or crab gender. The trends for crab size and gender among treatments opposed what might be expected if these factors were to have explained treatment differences in clam mortality.

The relative shell thickness of Manila clams might also have influenced the differences in clam mortality between treatments. However, as Figure 6 shows, there were no significant differences in the relationship between shell thickness and shell size (width) for early ($p > 0.60$) of late ($p > 0.20$) outplants treatments in 1997 with shell size explaining 1% or less of the variation in thickness overall ($R^2 = 0.003$ for early, $R^2 = 0.01$ for late). Therefore, difference in the timing of outplant did not produce changes in relative shell thickness.

In addition to the green crabs, there were other species of crabs recruiting into the clam bags including native shore crabs *Hemigrapsus oregonensis*, *Pachygrapsus crassipes*, and native *Cancer* spp. The *Cancer* spp. abundances were at very low abundance (<0.05 per bag) and *Pachygrapsus* were equivalently rare and unlikely to have had a measurable impact on the patterns. By contrast, *Hemigrapsus* were commonly found in the growout bags along with green crabs (overall 11.3 ± 6.9), although they rarely exceeded 20 mm in carapace width, and were also unlikely to have had a substantial impact on the number of crushed shells observed in the bags (Grosholz, unpubl. data). For example, the lowest *Hemigrapsus* abundance (mean per bag 7.30 ± 6.5) occurred in the outplant treatment with the highest clam mortality (March 1997) and the highest *Hemigrapsus* abundance (mean per bag 15.35 ± 6.34) occurred in the treatment with the lowest clam mortality in (March 1998, see Fig. 1).

We found that there were fewer *Hemigrapus* in 1997 when green crabs were more abundant, although the outplant date had opposing effects on *Hemigrapsus* abundance in different years. We also found a significant ($p < 0.001$) negative relationship overall between the abundance of *Hemigrapsus* and the abundance of green crabs (Fig. 4). Although adult *Hemigrapus* may prey on newly recruiting green crabs, the predatory relationship between these crabs may switch as green crabs approach adult size and prey on *Hemigrapsus* (Grosholz et al. 2000). The strong dependence of size as a determinant of intraguild predation among crabs has been amply demonstrated in western U.S. estuaries (Dumbauld et al. 1993, Iribarne et al. 1994).

In conclusion, our work demonstrated that decreased predation by green crabs on commercially produced Manila clams resulted from delaying the outplant timing of clams. We suggest that this low cost management tool could be used by Manila clam producers throughout western North America to reduce predation by green crabs in regions where green crabs are abundant. However, the specific timing of the delay needed to produce this result will likely vary with latitude, since the recruitment of green crabs is delayed with increasing latitude (Yamada, in press). Regardless, the Manila clam fishery would benefit by instituting low cost culture practices such as outplant delay, in order to avoid future losses to European green crabs.

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RECOVERY OF THE WESTERN ROCK LOBSTER, *PANULIRUS CYGNUS*, FROM EMERSION AND HANDLING STRESS: THE EFFECT OF OXYGEN CONCENTRATION DURING RE-IMMERSION

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ABSTRACT The effect of dissolved oxygen concentrations (110–120, 90–100, 70–80, 50–60, 30–40 and 10–20% saturation) on recovery of *Panulirus cygnus* from a period of activity/emersion was investigated. Biochemical disturbances induced in *P. cygnus* by emersion and handling included a large decrease in the hemolymph pH (0.7 units) and an increase in the hemolymph lactate concentration (2.44 mmol L^{-1}). Re-immersion resulted in very high rates of oxygen consumption (up to 2.5 times the pre-emersion rate), elevated hemolymph glucose (1.1–4.5 mmol L^{-1}) and further increases in hemolymph lactate (2.7–11.1 mmol L^{-1}), with actual concentrations dependent on the dissolved oxygen concentration. Water oxygen concentrations of 50–60% saturation or less slowed the rate of recovery; all lobsters recovering in water with 10–20% oxygen saturation died within 12 hours. Based on the rate of recovery of physiological parameters, lobsters should be allowed to recover for a minimum of eight hours between stress episodes, whilst maintaining an oxygen concentration of 70–80% saturation or above.

KEY WORDS: activity, live holding, emersion, oxygen consumption, oxygen concentration, *Panulirus cygnus*, recovery, stress

INTRODUCTION

During the live handling of western rock lobsters (*Panulirus cygnus*) after capture, they are subjected to many conditions that are likely to cause stress. They are often exposed to air during which time they may be handled. Their response is to attempt escape by strong beats of the tail (tail flicking). This resultant stress can be expressed quantitatively by biochemical changes (blood pH, glucose, lactate) (Spanoghe 1997) and by the development of an oxygen debt (Crear & Forteath 2001). It is presumed that when lobsters are re-immersed, they are able to recover from any physiological disturbances caused by air exposure (Taylor et al. 1997). A variety of processes take place during re-immersion—energy pools (phosphagen and ATP) are recharged, anaerobic end products are cleared from the tissues and pH disturbances are corrected. An organism-level manifestation of all of the above, essentially cellular processes of recovery, is a period of supranormal oxygen consumption (the oxygen debt) (Ellington 1983). Generally, physiological disturbances are fully reversed within 24 hours of re-immersion.

An adequate period of recovery in water is necessary after each stage of handling or live transport to avoid compounding the stress syndrome (Taylor et al. 1997). European lobsters, *Homarus gammarus*, deprived of the opportunity to recover after an episode of stress were in a significantly worse state after a subsequent period of air travel than lobsters that had been allowed to recover (Whiteley & Taylor 1992). Since time between episodes of stress is often limited during post-capture handling of *P. cygnus*, optimising the speed of recovery becomes important.

Inadequately designed holding systems, overstocking and natural variability in the oxygen concentration of intake water can lead to low oxygen concentrations in live holding tanks. Concentrations as low as 35% saturation have been measured in tanks on board boats (Crear & Forteath 1997). As stressed lobsters have a high demand for oxygen, its availability is likely to influence both the duration and extent of recovery. Minimum dissolved oxygen concentrations recommended for lobster holding systems vary from 40 to 80% saturation (Beard & McGregor 1991, Forteath et al. 1993, Boothroyd 1994), however there appears to be no biological rationale for those recommendations. Additionally, oxygen supersaturation has been suggested as a possible tool to aid the recovery of lobsters (Forteath 1995). This paper examines the effect of dissolved oxygen concentration on the recovery of *P. cygnus* from a stress episode.

MATERIALS AND METHODS

Lobster holding conditions and respirometry are outlined in Crear and Forteath (2000). All experiments were conducted at the acclimation temperature of 23°C, which is close to the mean annual temperature of inshore waters inhabited by juvenile *P. cygnus* (Chittleborough 1975). In view of the relationship between oxygen consumption (VO_2 – $mgO_2/g/h$) and *P. cygnus* wet body weight (Crear & Forteath 2001), a restricted weight range (367–515 g) was used in the experiments. The following specific methods were used for this series of experiments.

The study was performed in two experimental series. The first series studied the oxygen consumption of lobsters recovering from a period of stress. Lobsters were removed from the holding tank and emersed for 30 minutes at an air temperature of 23°C. Continual disturbance (handling) for the first 5 minutes was followed by disturbance every 5 minutes (lobsters were maintained in an open foam box when not being handled). Lobsters showed strong escape behaviors (tail flicking) during the initial period of disturbance. The response diminished as the emersion time increased and after 30 minutes emersion the lobsters were normally unresponsive to disturbance. Six to twelve lobsters were tested at each of six oxygen concentrations (110–120%, 90–100%, 70–80%, 50–60%, 30–40%, 10–20%). The dissolved oxygen concentration was controlled as in Crear and Forteath (2000). As *P. cygnus* has a nocturnal oxygen consumption rhythm (Crear & Forteath 2001), all experiments were commenced before 0900 hours to ensure that none of the measurement periods fell during the night. Rates of V_{O_2} were calculated immediately after placing the disturbed lobsters in the respirometers (0 h), and after re-immersion for 1, 2, 4,

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6, 8 and 24 hours. Ten lobsters were used to determine standard VO_2 (control oxygen consumption rate).

The second series of experiments consisted of measuring the hemolymph parameters of lobsters over the same time-period and under the same dissolved oxygen concentrations as above. Lobsters were removed from the holding tank and disturbed for 30 minutes before being placed in water of known oxygen concentration. Hemolymph samples were obtained immediately after the disturbance period (0 h), and after re-immersion for 1, 2, 4, 8 and 24 hours. Dissolved oxygen was maintained within 5% of the designated concentration (Crear & Forteath 2000). Lobsters were sampled only once during each experimental run. They were replaced in the holding tank for a minimum of 48 hours before being used again (Waldron 1991). Control hemolymph parameters were determined for 12 lobsters: four taken directly from the holding tank at 0900 hours, and two further groups of four tested 8 and 24 hours later.

Hemolymph Sampling

Prebranchial hemolymph was sampled (1 mL) from the infrabranchial sinus via the arthrodial membrane at the base of a walking leg (usually the 3rd or 4th pair). The sample was withdrawn with an ice-chilled 1 mL syringe (Luer -Tuberculin) using a 21 gauge (Luer -21G*1.5) needle. Hemolymph samples were obtained anaerobically to ensure minimum mixing with air, since changes in CO_2 equilibrium can alter pH values (Vermeer 1987). The hemolymph sample was taken within 20 seconds of lobster capture, and immediately placed into an ice-chilled 1-mL Eppendorf tube. A 250-μL aliquot was deproteinated by adding it to 500 μL of ice-chilled 0.6 mol L^{-1} perchloric acid (PCA). The deproteinated sample was snap-frozen in liquid nitrogen and stored at −86°C for later measurement of lactate and glucose. The hemolymph remaining in the original tube was used for measuring pH.

Hemolymph Analyses

Hemolymph pH was measured with a calomel electrode (Activon Semi-Micro AEP336) connected to a pH meter (WTW pH 323). The probe was calibrated in buffer solutions chilled to the same temperature as the hemolymph samples (0°C). pH at 0°C varies from pH *in vivo* at ambient temperatures (0.49 units higher than at 23°C, Crear 1998), but this was an essential concession to retard clot formation (Vermeer 1987); clots formed within 5 minutes at ambient temperature.

The deproteinated hemolymph samples were centrifuged at 8000 g for 3 min. The supernatant (600 μL was generally obtained) was neutralized with 3 mol L^{-1} KOH (6.4 μL per 600 μL). The samples were stored on ice for 15 minutes before being centrifuged at 8000 g for 3 min so that the perchlorate precipitate could be removed. The supernatant (~550 μL) was either frozen (−86°C) for later analysis or analyzed immediately for lactate and glucose.

Lactate concentrations were determined enzymatically with the Boehringer-Mannhein analysis kit (Cat. No. 139084). The absorption was measured at 340 nm on a GBC UV/VIS 916 spectrophotometer. Glucose concentrations were determined with a Sigma glucose test kit (No. 510), which is based on the glucose-oxidase method. The absorption was measured at 450 nm on a GBC UV/VIS 916 spectrophotometer. New calibration curves were made up for each sample run and all assays were run in duplicate.

Statistical Analyses

Student's *t*-test was used to test for significant differences ($P<0.05$) between control and recovery values. Where appropriate, Student's *t*-test for samples with unequal variances was used. The one-way ANOVA was used to test for differences between treatments at each time-period. The Levene test was used to test for homogeneity of variance, and where necessary an appropriate transformation was performed before further analysis. Following ANOVA, means were compared by Tukey-HSD test. Correlation analysis was used to analyse the relationship between dissolved oxygen concentrations and lactate changes. The critical oxygen tension (P_c) was calculated (Cochran & Burnett 1996, Crear & Forteath 2000) from the oxygen consumption data immediately after re-immersion. All analyses were performed on the SPSS statistical package with α set at 0.05. All values are expressed as mean ± S.E.

RESULTS

There was no significant difference in the parameters measured for the control lobsters at 0, 8 and 24 hours, therefore the data were pooled. Survival was 100% in all treatments except for the 10–20% oxygen saturation, where no animals survived for more than 12 hours.

Oxygen consumption

In all treatments, apart from the 10–20% treatment, VO_2 was significantly higher after re-immersion than the control VO_2. From those high values, VO_2 reduced slowly over time (Fig. 1), with the pattern of recovery varying with the oxygen concentration. At high concentrations (90–100% and 110–120% oxygen saturation), VO_2 was not significantly different to the control VO_2 after eight hours re-immersion. In all other treatments recovery of VO_2 to control levels took longer, and in the 30–40% treatment, VO_2 remained significantly higher than the control VO_2 over the 24-hour re-immersion period. Lobsters in the 10–20% oxygen saturation treatment had significantly lower VO_2 than the control VO_2 at each measurement period.

Upon re-immersion, VO_2 rates varied with the oxygen concentration, being highest for the 110–120% and 90–100% oxygen saturation treatments (Table 1). At lower oxygen saturations, VO_2 decreased significantly with the dissolved oxygen concentration. From this data, P_c was calculated to be 63.1%.

The amount of excess oxygen consumed over the control rate during recovery also varied with oxygen concentration (Table 2). Lobsters recovered in water containing 70–80% oxygen saturation or higher consumed the least amount of oxygen during the recovery period. In comparison, lobsters in the 50–60% treatment consumed 1.3 times as much oxygen during the initial eight-hour recovery period and approximately twice as much oxygen in achieving full recovery (see Note b in Table 2). Lobsters in the 30–40% treatment consumed 0.75 times as much oxygen during the initial eight-hour recovery period, but had consumed 1.6 times as much oxygen after 24 hours although they had still not achieved full recovery.

Hemocymph parameters

Lobster hemolymph pH decreased significantly during the 30-minute disturbance period, from control concentrations of 8.36±0.01 to 7.66±0.03. In general, the pH returned to, or close to, the control concentrations after four hours of being re-immersed. However, during the first hour of re-immersion two distinct recovery patterns were noted: (a) at oxygen concentrations of 70–

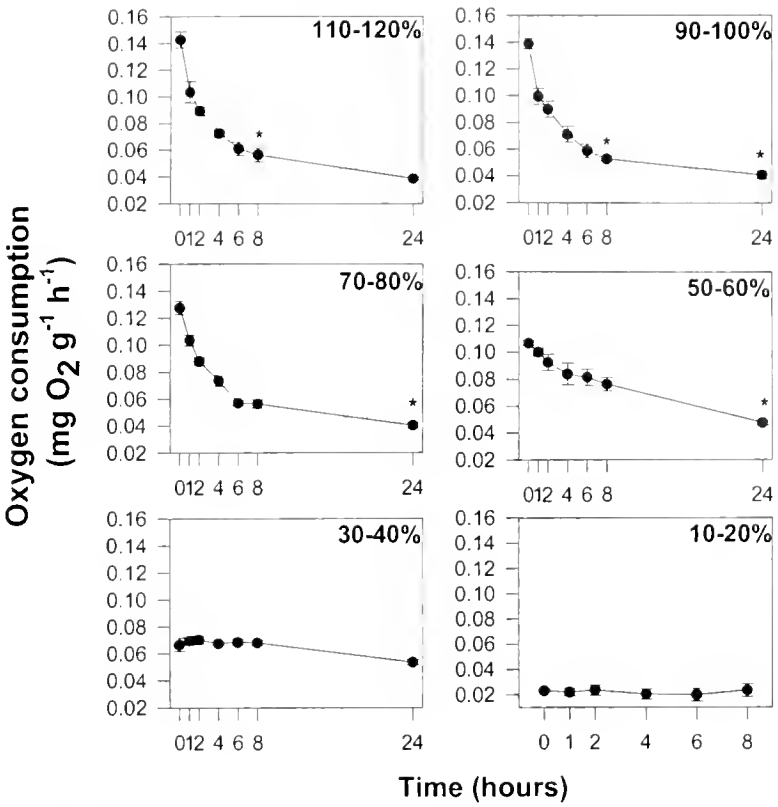

Figure. 1. Oxygen consumption (mg O_2 g^{-1} h^{-1})(mean ± SE) of the western rock lobster, *Panulirus cygnus*, over a 24-h period during "recovery" from disturbance (n = 6 to n = 12). The lobsters were disturbed (emersed and handled) for 30 minutes prior to re-immersion in water containing different concentrations of dissolved oxygen. Dotted line: control oxygen consumption rate (n = 10).*, not significantly different to the control. The lines are drawn for ease of viewing.

80% and higher the pH remained at the low level measured after the emersion period; whilst (b) at lower oxygen concentrations the pH increased markedly (Fig. 2, Table 3).

In some treatments (70–80, 50–60 and 30–40%) the pH increased to a significantly higher level than that of the control during the recovery period, however in all those treatments the pH was not significantly different to the control after 24 hours. The pH of lobsters in the 110–120% treatment was significantly lower than the control, and all other treatments, after 24 hours.

Hemolymph lactate increased significantly during the 30-minute disturbance period, from a resting concentration of 0.05 ± 0.02 mmol L^{-1} to 2.44 ± 0.37 mmol L^{-1} (Fig. 3). Lactate increased further during the first hour of re-immersion with the largest increases occurring at the lower oxygen concentrations (Table 4; Fig.

TABLE 1.

The results of the ANOVAs comparing oxygen consumption of lobsters (*Panulirus cygnus*) in each oxygen concentration treatment at each measurement time during the 24 h recovery period. Different letters denote significantly different results.

Oxygen Concentration (%)	Recovery Time (h)						
	0	1	2	4	6	8	24
110–120	a	a	a	ab	b	bc	b
90–100	a	a	a	ab	b	c	b
70–80	b	a	a	ab	b	bc	b
50–60	c	a	a	a	a	a	a
30–40	d	b	b	b	ab	ab	a
10–20	e	c	c	c	c	d	N/A

N/A - the lobsters in this treatment did not survive for 24 h.

TABLE 2.

The amount of excess oxygen consumed (mg O_2 g^{-1}) over the control rate during recovery lobsters (*Panulirus cygnus*) from emersion and handling. Oxygen consumed during 8-h and 24-h of recovery are shown.

Time	Dissolved Oxygen Concentration (%)					
	10–20	30–40	50–60	70–80	90–100	110–120
8 h	−0.163	0.184	0.334	0.251[c]	0.253	0.266
24 h	N/A	0.425[a]	0.593[b]	N/A	N/A	N/A

N/A not applicable to this time period at that particular dissolved oxygen concentration

[a] Oxygen consumption was still significantly higher than the control after 24 h so the total oxygen consumed during recovery would be slightly higher than this value.

[b] Oxygen consumption may have returned to the control prior to the 24 h period so this value may be an overestimation.

[c] Although the oxygen consumption was still significantly higher than the control after 8 h it was not significantly different to either the 110–120% or 90–100% rate. Therefore, the total amount of oxygen consumed was only calculated up to the 8 h mark.

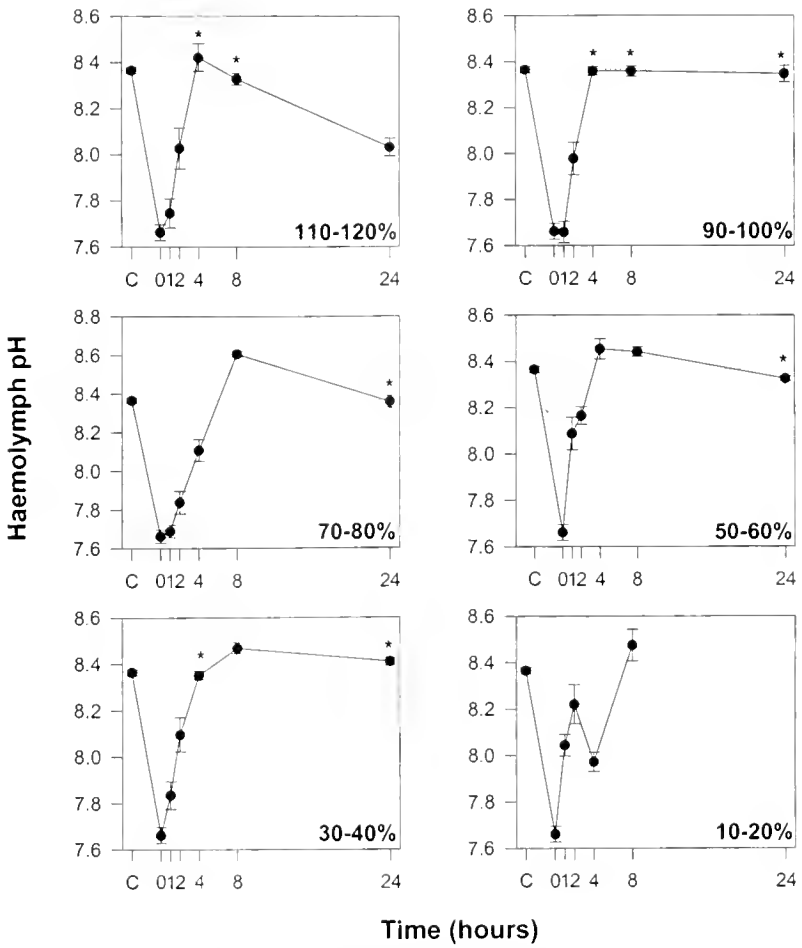

Figure. 2. Hemolymph pH (mean ± SE) of the western rock lobster, *Panulirus cygnus*, over a 24-h period during "recovery" from disturbance (n = 6 to n = 12). The lobsters were disturbed (emersed and handled) for 30 minutes prior to re-immersion in water containing different concentrations of dissolved oxygen. Dotted line: control hemolymph pH value (n = 12).*, not significantly different to the control (C). The lines are drawn for ease of viewing.

3). The increase was correlated (P = 0.005) with oxygen concentration (Fig. 4).

From those high values, lactate reduced slowly over time (Fig. 3), with the pattern of recovery varying with the oxygen concentration. At high oxygen concentrations (70–80%, 90–100% and 110–120% saturation) lactate was not significantly different to the control after eight hours re-immersion. In the other treatments, lactate remained high for longer, and in the 30–40% treatment lactate was still significantly higher than the controls after 24 hours re-immersion. The lactate concentration of lobsters in the 10–20% treatment remained very high.

TABLE 3.

The results of the ANOVAs comparing the hemolymph pH of lobsters (*Panulirus cygnus*) in each oxygen concentration treatment at each measurement time during the 24-h recovery, period. Different letters denote significantly different results.

Oxygen Concentration (%)	Recovery Time (h)					
	0	1	2	4	8	24
110–120	a	bc	abc	a	c	b
90–100	a	c	bc	a	c	a
70–80	a	bc	c	b	a	a
50–60	a	a	ab	a	b	a
30–40	a	b	ab	a	b	a
10–20	a	a	a	b	b	N/A

N/A - the lobsters in this treatment did not survive for 24 h.

Hemolymph glucose increased during the 30-minute disturbance period from 0.35 ± 0.06 mmol L^{-1} to 0.44 ± 0.06 mmol L^{-1}, but it was not a significant increase (Fig. 5). After 1-hour re-immersion, the glucose concentration in all treatments was significantly higher than the control. The largest increases were measured in the low oxygen saturation treatments (Table 5). In general, the high glucose concentrations were maintained for between two and four hours, most were not significantly different to the controls after eight hours re-immersion. Lobsters in the 110–120% and 50–60% treatments still had significantly higher concentrations than the controls after eight hours re-immersion, however the concentrations were not significantly different to those in all other treatments except for the 10–20% treatment.

DISCUSSION

Biochemical disturbances induced in *P. cygnus* by emersion and handling included a large decrease in the hemolymph pH and an increase in the hemolymph lactate concentration. Re-immersion

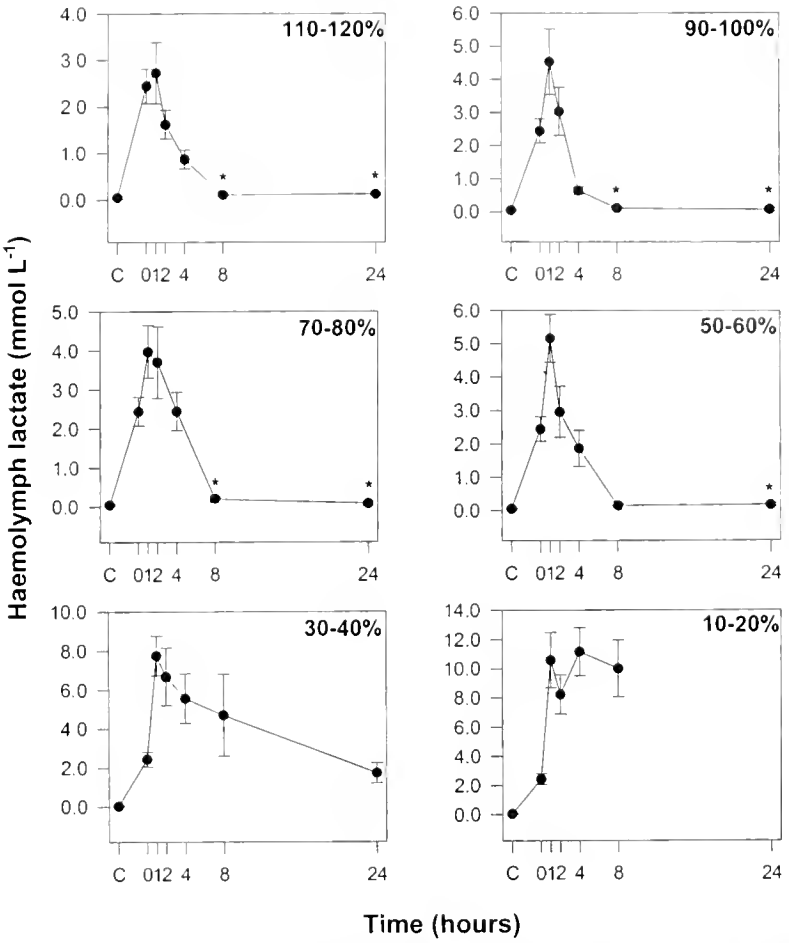

Figure. 3. Hemolymph lactate concentration (mmol L^{-1})(mean ± SE) of the western rock lobster, *Panulirus cygnus*, over a 24-hour period during "recovery" from disturbance (n = 6 to n = 12). The lobsters were disturbed (emersed and handled) for 30 minutes prior to re-immersion in water containing different concentrations of dissolved oxygen. Dotted line: control hemolymph lactate concentration (n = 12). *, not significantly different to the control (C). The lines are drawn for ease of viewing.

was followed by very high rates of oxygen consumption, elevated hemolymph glucose concentrations and further increases in the hemolymph lactate concentrations. The dissolved oxygen concentration during re-immersion influenced both the rate and the form of recovery from the physiological disturbances, with oxygen con-

TABLE 4.

The results of the ANOVAs comparing the haemolymph lactate concentration (mmol L^{-1}) of lobsters (*Panulirus cygnus*) in each oxygen saturation treatment at each measurement time during the 24-h recovery period. Different letters denote significantly different results.

Oxygen Concentration (%)	Recovery Time (h)					
	0	1	2	4	8	24
110–120	a	c	b	d	c	b
90–100	a	c	b	d	c	b
70–80	a	c	b	c	c	b
50–60	a	bc	b	cd	c	b
30–40	a	ab	a	b	b	a
10–20	a	a	a	a	a	N/A

N/A - the lobsters in this treatment did not survive for 24 h.

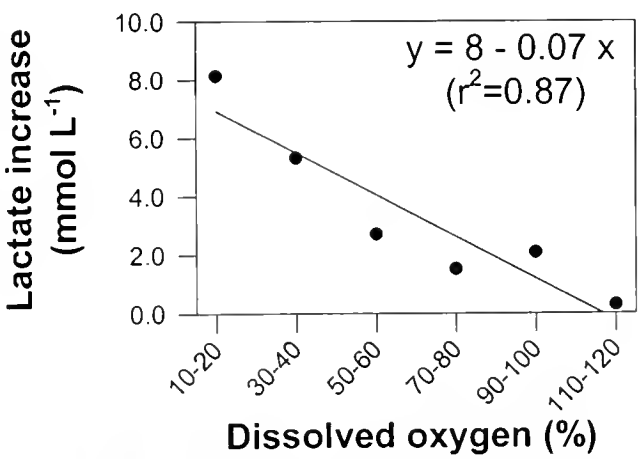

Figure. 4. Increase in hemolymph lactate (mmol L^{-1})(●) during the first hour of recovery after re-immersion of lobsters, *Panulirus cygnus*, in water at different dissolved oxygen concentrations. The equation describes the relationship.

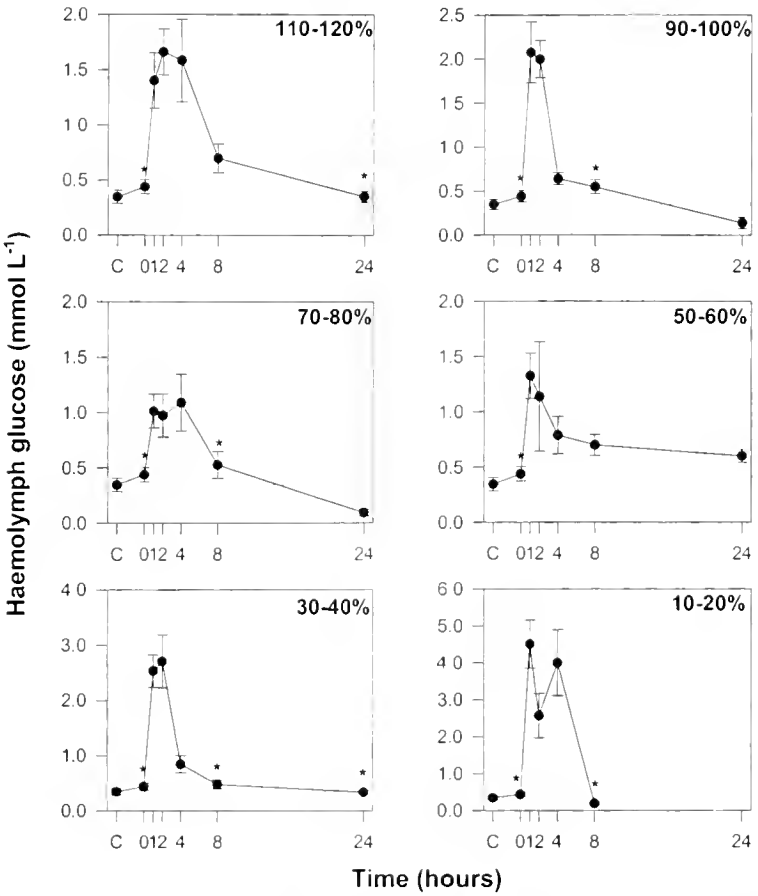

Figure. 5. Hemolymph glucose concentrations (mmol L^{-1}) (mean ± SE) of the western rock lobster, *Panulirus cygnus*, over a 24-hour period during "recovery" from disturbance (n = 6 to n = 12). The lobsters were disturbed (emersed and handled) for 30 minutes prior to re-immersion in water containing different concentrations of dissolved oxygen. Dotted line: control hemolymph glucose concentration (n = 12). *, not significantly different to the control (C). The lines are drawn for ease of viewing.

centrations of 70–80% saturation or above maximizing the rate of recovery. At those concentrations stress indicators returned to normal levels within eight hours of re-immersion. Oxygen concentrations <60% saturation, which are commonly measured in holding tanks (Crear & Forteath 1997), slowed the rate of recovery.

The aerobic response of *P. cygnus* during recovery follows a typical Type V pattern. That is, the oxygen consumed during recovery exceeds the predicted aerobic oxygen deficit (Herreid II 1980). If it is assumed that the emersed lobsters can take up ~50% of the oxygen that they are able to take up in water (Whiteley & Taylor 1990, Waldron 1991), and that they were fully active over the 30 minute period of disturbance, then the maximum oxygen deficit would be ~0.06 mg O_2 g^{-1}. In fact, this is less than one-quarter of the oxygen debt incurred at high concentrations dissolved oxygen. Suggested uses for the excess oxygen include: (1) metabolising anaerobic end products; (2) re-establishing resting oxygen concentrations in body tissues; (3) replenishing high-energy phosphate reserves; and (4) meeting energy costs associated with increased branchial chamber ventilation and hemolymph circulation (Herreid 1980, Head & Baldwin 1986).

TABLE 5.

The results of the ANOVAs comparing the haemolymph glucose concentration (mmol L^{-1}) of lobsters (*Panulirus cygnus*) in each oxygen saturation treatment at each measurement time during the 24-hour recovery period. Different letters denote significantly different results.

Oxygen Saturation (%)	Recovery Time (h)					
	0	1	2	4	8	24
110–120	a	cd	bcd	b	a	b
90–100	a	bc	abc	b	a	c
70–80	a	d	d	b	a	c
50–60	a	cd	cd	b	a	a
30–40	a	b	a	b	ab	b
10–20	a	a	ab	a	b	N/A

N/A the lobsters in this treatment did not survive for 24 h.

The recovery period at high concentrations of dissolved oxygen (70–80% and above) was similar to that observed previously for *P. cygnus* (Crear & Forteath 2001) and for other crustaceans (McMahon et al. 1979, Waldron 1991). However, the recovery period increased as the oxygen concentration decreased, indicating that the lobsters either (a) were not accessing sufficient oxygen to repay the debt as quickly or (b) were increasing the size of the debt as a result of the processes involved in repaying the debt or (c) both. Thus:

(a) The ability of lobsters to take up oxygen upon re-immersion was dependent on the dissolved oxygen concentration. The critical oxygen tension (P_c) in this study (63.1%) was close to the 62.8% calculated by Crear and

Forteath (2001) for active *P. cygnus* at 23 C. The aerobic scope for activity (the amount of oxygen available to the lobsters above normal maintenance requirements) increased as oxygen concentrations increased up to P_c. Lobsters with a large aerobic scope for activity should be able to shorten the time required to repay a similar oxygen debt. Lobsters in the 30–40% treatment were very limited in the amount of oxygen they were able to extract from the water (≈50% of maximum VO_2). Therefore, total oxygen consumption over the first eight hours of re-immersion in that treatment was ≈25% lower than in the higher oxygen treatments. Access to oxygen was a major problem with these lobsters and would explain (at least partly) the slow recovery rates. Similarly, in response to an injection of lactate *C. maenas* increased VO_2, but the response was lesser, and lasted longer, under hypoxic conditions than under normoxic conditions (De Wachter et al. 1997). Those authors suggested that the response was due to the larger aerobic scope at the higher oxygen concentration.

(b) The total amount of oxygen consumed in the 50–60% treatment during the first eight hours of re-immersion was ~30% higher than in the treatments with higher oxygen concentrations. Although the oxygen consumed was high, they still had not repaid the oxygen debt, which indicates that there were increased energetic costs associated with recovery at that oxygen concentration. The increased energetic costs could arise from: (i) the increased cost of branchial chamber ventilation and hemolymph circulation when diffusion of oxygen from the external medium to the hemolymph via an oxygen gradient would be minimal and/or, (ii) an increased reliance on anaerobic respiration, together with production of lactate and associated energetic costs of resynthesizing the substrate or oxidizing lactate further to carbon dioxide (Gäde et al. 1986).

The pH response of crustaceans to the combination of stressors used in this study (emersion, exercise and handling) has not often been investigated. Spanoghe (1997) recorded a similar large decrease (0.7 pH units) in *P. cygnus* after one hour of emersion and handling. A pH change of that magnitude must be considered a large physiological perturbation (Vermeer 1987). Return to control pH values after re-immersion was optimized at oxygen concentrations of 90–100% and above. The time taken was similar to that of other crustaceans (McDonald et al. 1979, Waldron 1991, Whiteley & Taylor 1992).

Schmitt and Uglow (1997) concluded that CO_2 accumulation was mainly responsible for emersion-induced acidosis in *Nephrops norvegicus*, despite the presence of high concentrations of lactate. Recovery of acid-base status during re-immersion in this study varied with the oxygen concentration, and also appears not to be determined by lactate concentrations. Lobsters at low oxygen concentrations (50–60%, 30–40% and 10–20%) had large increases in lactate during the first hour of re-immersion, while their hemolymph pH showed significant increases. Taylor and Wheatly (1981) noted that the potential acidosis, which the increase in lactate represents, was overridden by a respiratory alkalosis due to the washout of CO_2 during the period of hyperventilation. In the present study, oxygen consumption upon re-immersion was relatively low at lower oxygen concentrations, so less CO_2 would have been produced, whilst high ventilation and perfusion activities would have promoted the excretion of CO_2 across the gills. These two factors may have resulted in the large pH increase after one hour of re-immersion into poorly oxygenated water. At the higher dissolved oxygen concentrations, the hemolymph pH remained low after one hour of recovery, even though the hemolymph lactate was lowest in these lobsters. The high V_{O_2} of lobsters held at the higher oxygen concentrations may result in CO_2 concentrations remaining elevated in the hemolymph, thus helping to maintain a low pH during the initial stages of recovery. Elimination of accumulated CO_2 is usually rapid, however, Waldron (1991) found that hemolymph CO_2 partial pressure remained significantly elevated for two hours during re-immersion after a period of emersion and exercise in *J. edwardsii*.

A hemolymph alkalosis was measured during the recovery period in lobsters subjected to 70–80% oxygen saturation or lower. Crustaceans generally hyperventilate in response to hypoxia, leading to hypocapnic alkalosis due to an increase in the rate of excretion of CO_2 (Hagerman & Uglow 1985). The pH of *A. leptodactylus* increased by 0.16 units with exposure to 30% oxygen saturation (Sinha and Dejours 1980). In other studies in which the pH of crustaceans undergoing recovery has risen (Truchot 1975; Whiteley and Taylor 1992; Spanoghe 1997), the oxygen concentrations in the recovery tanks may have been lower than optimal.

Resting concentrations of hemolymph lactate (0.05 mmol L^{-1}) were similar to those measured in other decapod crustaceans (0.14 mmol L^{-1} for *J. edwardsii*, Waldron 1991; 0.14 mmol L^{-1} for *Ranina ranina*, Paterson et al. 1994; 0.09 mmol L^{-1} for *Carcinus maenas*, De Wachter et al. 1997). There have been few reports on the hemolymph lactate concentrations of crustaceans that have been emersed and exercised. However, in *J. edwardsii* after a short period of exercise followed by one hour of emersion lactate increased by ~1.0 mmol L^{-1} (Waldron 1991) and in *P. cygnus* lactate increased by 2 mmol L^{-1} after 40 minutes of emersion and disturbance (Spanoghe 1997); increases which were similar to this study. The slow rate of lactate removal from the hemolymph indicates that *P. cygnus*, like many other crustaceans, lack the means for rapid removal of lactate (McDonald et al. 1979, Ellington 1983, Waldron 1991). Return to normal concentrations was optimized at oxygen concentrations of 50–60% and above.

The increase in hemolymph lactate concentration of *P. cygnus* indicates that it was unable to maintain an adequate supply of oxygen to the tissues during the period of disturbance and needed to rely, at least partially, on anaerobic metabolism to supply its energy requirements (Spicer et al. 1990). The rise in lactate concentration after re-immersion has also been noted in other crustaceans subjected to periods of exercise and/or emersion (McDonald et al. 1979, Taylor & Wheatly 1981, Whiteley and Taylor 1992). Increased hemolymph lactate concentrations after re-immersion may be due to the release of lactate previously stored in the tissues during the disturbance period, as suggested by Taylor and Wheatly (1981) and Waldron (1991). In *C. destructor* it appears that a steady state between tail muscle and hemolymph lactate pools is reached quite rapidly (Head & Baldwin 1986), hence lactate release may not fully explain the increased concentrations. Another possible explanation is that lactate production may have increased on re-immersion due to a high energy demand requiring a contribution from both aerobic and anaerobic metabolism (Head & Baldwin 1986, Gruschczyk & Kamp 1990, Whiteley & Taylor 1992). Onnen and Zebe (1983) suggested that the use of anaerobic metabolism during the recovery process might ensure that the muscle function is restored as soon as possible. In this study, the relative increase in the lactate concentration during the first hour was dependent on the dissolved oxygen concentration in the water. When oxygen could not fully fuel the aerobic portion of the energy requirements of recovery, the shortfall was made up via anaerobic

metabolism. This suggests that the observed increase in hemolymph lactate is probably due to the continued use of anaerobic energy sources after re-immersion, rather than the release of sequestered lactate. During recovery in the 110–120% treatment, metabolism appears to be mainly aerobic as shown by the absence of further accumulation of lactate.

Hemolymph glucose concentrations of control lobsters are similar to those measured in other studies: 0.2–0.3 mmol L^{-1} for *Nephrops norvegicus* (Spicer et al. 1990, Schmitt & Uglow 1997); 0.2–0.4 mmol L^{-1} for *P. cygnus* (Tod & Spanoghe 1997). The maximum concentrations of hemolymph glucose measured in this study (1.0 to 4.5 mmol L^{-1}) also covered the range of maximum concentrations measured by those researchers. As has been noted in other studies (Onnen & Zebe 1983, Gruschczyk & Kamp 1990, Tod & Spanoghe 1997), there was a marked hyperglycemia in the hemolymph of *P. cygnus* one hour after re-immersion. In this study, the hyperglycaemia was more severe in lobsters subjected to low dissolved oxygen concentrations, suggesting that more energy substrate was required because the lobsters were relying more on anaerobic metabolism. When aerobic mechanisms of energy production are impaired, in order to provide a given amount of energy, more glucose must undergo anaerobic glycolysis (Storey & Storey 1990), as anaerobic glycolysis produces only about 1/20th of the energy produced via aerobic glycolysis (Eckert et al. 1988). During recovery in this study, anaerobic glycolysis (as indicated by lactate concentration), increases as oxygen saturation decreases. Therefore, the increases in hemolymph glucose concentrations would be expected.

The time period of recovery from hyperglycaemia has not been well studied, but it was similar to that for *P. cygnus* in Spanoghe's (1997) study, and to that recorded for *Palaemon serratus* and *P. elegans* after a period of emersion (Taylor & Spicer 1987). Speed of recovery to normal glucose concentrations was optimized at oxygen concentrations of 70–80% and above.

In conclusion, oxygen has a considerable effect on the recovery response of *P. cygnus*. The duration and the effectiveness of the recovery process are of great functional importance. Recovery from anaerobic metabolism should be sufficiently rapid and complete for the organism to cope with further periods of stress. For example, in the case of muscles powering escape responses, this process of recovery must be sufficient to allow the organism to evade predators (Ellington 1983). Using speed of recovery as the criteria for evaluating the effectiveness of oxygen concentrations, the results show that a minimum of 70–80% saturation is required, with some indication that higher concentrations (including slight supersaturation) may offer some benefits.

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EFFECT OF DIETARY CARBOHYDRATES ON GLUCONEOGENESIS IN PREMOLT *LITOPENAEUS STYLIROSTRIS* JUVENILES AND PRE ADULTS

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ABSTRACT Because in the premolt stage *L. stylirostris* should produce glycogen to prepare chitin synthesis, the present paper was directed to answer the following questions: (a) Could an absence of dietary carbohydrates (CHO) enhance the gluconeogenesis pathway in premolt shrimp, following the trend in species of carnivorous fish? (b) In premoulting shrimp is it necessary to force the glyconeogenic pathway through intensive feeding or is a two meal/day feeding schedule enough? (c) Is the CHO metabolism adaptation affected by the age of shrimp? and (d) are the same mechanisms working in moult stage C and D? A comparison with shrimp fed the same diets and in stage C has been done to determine the effect of the molt stage and dietary CHO levels in glycogen accumulation of this shrimp species. Results obtained showed that in stage D shrimp the gluconeogenic pathway was enhanced, both in an intensive or in a two meal/day feeding schedule, independently of the age of the shrimp. The accumulation of glycogen to support both chitin synthesis and metabolic glucose is proposed as an explanation. Comparing the results obtained in the present paper with that of *L. stylirostris* juveniles published earlier, it is possible to observe that both in 12 hour fasting and during glycemia, the glycogen concentration in stage C shrimp was opposite of that observed in stage D shrimp. These results show that the intensity of the gluconeogenesis pathway changes with the molting stage, being of more importance in the premolt stage (D) than during the intermoult stage (C).

KEY WORDS: carbohydrate metabolism, gluconeogenesis, Digestive gland, blue shrimp *Litopenaeus stylirostris*, molt stage, juveniles, glycogen

INTRODUCTION

Carbohydrate metabolism is complex in Crustaceans. Although metabolic aspects are identified, the whole aspect of regulation is still uncertain (Shiau & Peng 1992, Ceccaldi 1998). In recent papers *L. stylirostris* juveniles showed a limited capacity to use dietary carbohydrates. A saturation curve was measured in glycogen levels and α-amylase activity against dietary carbohydrate levels (CHO). Maximum values of both the metabolic indexes were obtained in shrimp fed diets containing 21% CHO and that was recommended as a maximum shrimp dietary CHO level for *L. stylirostris* culture (Rosas et al. 2001a).

This limitation may be a consequence of a metabolic adaptation to use protein as a primary source of energy. Protein is the main reserve substrate in shrimp, and can be converted to carbohydrates following the gluconeogenic pathway (Campbell 1991). Recently we had observed that the gluconeogenic pathway is at constant rate in several shrimp species. In *L. stylirostris* (Rosas et al. 2000), *L. setiferus* (Rosas et al. 2001b), and *L. vannamei* (Taboada et al. 2001) a relatively high glycogen level (4.3 mg/g, 4.9 mg/g, and 10.1 mg/g) was observed in shrimp fed during 40 days with 1% of dietary CHO levels, indicating the importance of gluconeogenesis. The internal ammonia, ammonia excretion, glutamate dehydrogenase activity (GDH), and osmotic pressure measurements showed that the route of protein metabolism was purposed as a donor of amino acids to obtain glycogen. Gluconeogenesis in crustaceans is a biosynthetic pathway for *de novo* synthesis of glucose from lactate or alanine in an inter-tissue cooperation pathway. According to Lallier and Walsh (1991), the flux of lactate and alanine is oriented to glucose biosynthesis intensively, giving at the digestive gland a gluconeogenic role. An elevation in phosphoenolpyruvate carboxykinase (PEPCK), the regulatory enzyme of gluconeogenesis pathway, was related to the increase of gluconeogenesis pathway in *L. vannamei* fed with 1% dietary CHO (Rosas et al. 2001a). Similar results were seen in *Chasmagnathus granulata* crabs (Oliveira & Da Silva 1997) and in *Callinectes sapidus* (Lallier & Walsh 1991). Those results emphasized the gluconeogenetic role of the digestive gland.

Shrimp, like other decapods, have an organic reserve cycle which show modifications. During the molt cycle, glycogen reserves are actively accumulated from stage Do, preparing the animal for chitin synthesis (Renaud 1949). If the shrimp can synthesize glycogen during stage C, the mechanisms associated with glycogen synthesis in stage Do to D1‴should be overstimulated, producing important changes at the tissue level.

Hepatosomatic index (or Digestive gland index; DGI) has been used as an index in CHO metabolism of the digestive gland, because there is a direct relation between glycogen concentration and digestive gland weight in shrimp (Gibson & Barker 1979, Jayaprakas & Sambhu 1998, Ramos et al. 1996, Sambhu & Jayaprakas 1997). According to Renaud (1949), this DGI should be changed according to the molt stage, showing the changes in CHO metabolism associated with storage reserves for molting. To date, CHO metabolism has been studied in stage C shrimp but we do not know if the gluconeogenic pathway in shrimp works in the same way when it needs more CHO to be prepared for chitin synthesis.

In carnivorous fish, (cod fish) fed without dietary CHO, it was observed that the hepatosomatic index was higher than that observed in fish fed with 25–50% dietary CHO (Hemmre et al. 1990). This was interpreted as an adaptation of cod to increase the gluconeogenic pathway to store CHO reserves. Is this type of mechanism working in the same form in shrimp? There is no information in penaeid shrimp.

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Glycemia produced by meal has been reported in several shrimp species. Shiau and Peng (1992) reported that glycemia in *P. monodon* was produced two to five hours after feeding and depended on the CHO type. Similar results were reported by Abdel Rahman (1996) in *P. japonicus*, by Rosas et al. (1995a) in *L. setiferus* adult males, by Lignot et al. (1999) in *L. stylirostris* and by Cousin (1995) and Rosas et al (2001a) in *L. vannamei*. Once the food is digested in the gut, chime and fine particles are digested in the lumen and absorbed by diffusion by the inner portion of the digestive gland tubules, initiating the accumulation of reserves. After absorption, glucose passed through the digestive gland (DG) wall to the blood and glycogen is stored (Al-Mohanna & Nott 1987). This mechanism should be modified when shrimp are fed without CHO. In first instance it should be fast enough to transform the dietary protein into glycogen during intermoult period without CHO supply. In *L. setiferus* and *L. vannamei* intermoult (stage C) shrimp fed without CHO, it was observed that during glycemia, glycogen is accumulated two to three hours after feeding, evidencing that without dietary CHO glycemia is a fast process (Rosas et al. 2001b). In premolt shrimp (stage D), this mechanism could be enhanced.

Most experiments done with nutrients tend to test a gradient of a nutrient (protein for example) against another (carbohydrate CHO for example). Then interpretation leads to a double sense whether one considers one nutrient in relation with another. To alleviate that problem, diets can be formulated and distributed on a pair-feeding approach, which can maintain protein constant with and without dietary CHO level. With this method it is possible to maintain the shrimp in a constant feeding schedule causing a permanent glycemia (Cousin 1995, Rosas et al. 2000a).

Because in the premolt stage, shrimp should produce glycogen to prepare for chitin synthesis, the present paper was directed to answer the following questions: (a) Could an absence of dietary CHO enhance the gluconeogenesis pathway in premolt shrimp, following the trend in species of carnivorous fish? (b) In premolting shrimp is it necessary to force the glyconeogenic pathway through an intensive feeding approach or is it enough to use a two meal/day feeding schedule? and (c) Are the CHO metabolism adaptations affected by the age of shrimp? A comparison with shrimp fed with the same diets and in stage C (Rosas et al. 2000) was done to determine the effect of the molt stage and dietary CHO levels in glycogen accumulation of this shrimp species.

Digestive gland index, digestive gland glycogen concentration, and blood glucose levels of juveniles and adults of *L. stylirostris* were evaluated. Two experiments were done at the "Centre Océanologique du Pacifique" (Ifremer) located in Tahiti. In the first experiment the effect of dietary CHO levels on digestive gland glycogen, DGI, and blood glucose after glycemia produced by feeding in premolt shrimp were evaluated. The second experiment determined the effect of age of premolt shrimp on DGI and digestive gland glycogen after intensive feeding and in the absence of dietary CHO.

MATERIALS AND METHODS

Animals

First Experiment

One hundred shrimp (9.45 ± 0.15 g wet weight) were reared for 15 to 18 days at a density of 8 shrimp/72-L (8 shrimp/m^2) tank in a flow through sea water system (1 L/min; salinity of 35‰). In such system seawater was renewed 15 times a day. The light dark photoperiod was 12 h/12 h and water temperature was 28 + 1°C. The shrimp were fed *ad libitum* three times a day (0800, 1200, and 2000). Uneaten food particles were removed each day after each meal. Four tanks were randomly assigned to each carbohydrate level.

Second Experiment

Fifty juvenile shrimp (7.8 ± 0.5 g wet weight) and fifty pre adult shrimp (19.4 ± 1.9 g wet weight) were reared in 72-L dark tanks at a density of 6 shrimp/tank (6 shrimp/m^2) in a flow through sea water system (35‰) and with a water exchange of 45 L/h. The light dark photoperiod was 12 h/12 h and water temperature was 28 ± 1°C. The amount of the diet fed was calculated to have the same amount of protein ingested and feeding frequency was adjusted accordingly. High CHO (HCHO) level diet was given seven times a day at the same intervals in a quantity of 0.07 g/animal per time. This time allow let shrimp time to consume 100% of the food. The low CHO (LCHO) level was adjusted to represent 60% of the amount ingested by animals receiving the HCHO dietary level. To do that, one out of two feed distributions was omitted in the LCHO level. Shrimp were fed 12 days for juveniles and 8 days for pre adults. This period was considered to have each sample population receiving a given experimental diet and to have animals in intermolt stage comprised between Do and D1‴.

Diets

First Experiment

The juveniles were fed formulated semi purified diets prepared with different levels of carbohydrates: 1%, 10%, 21%, and 33%. The ingredients composition of the experimental diets are presented in Table 1. The experimental diets were prepared by thoroughly mixing the dry ingredients with oil and then adding water until a stiff dough resulted. The dough was then passed through a mincer with a die, and the resulting spaghetti-like strings were air dried at 60 C. After drying, the diets were broken up, sieved to a convenient pellet size, and stored at −4°C.

Second Experiment

Experimental diets were formulated as described in Table 1. These diets were prepared similarly to the diets used in the first experiment.

Moulting Stages

In both experiments, the molt stage was observed at the uropodae with a binocular (×50) for the largest characteristics and the microscope (×400) for the smallest characteristics, following the criteria published by Drach and Tchenigovtzeff (1967) for decapods and Aquacop (1973) for shrimp. In the present paper only shrimp in stages Do to D1‴ were used.

Physiological Measurements

Digestive Gland Index

At the end of the feeding period of both experiments, shrimp were sacrificed and the digestive gland index was measured (DGI). The digestive gland weight was obtained by using a Mettler balance (±0.001 g) to get wet weight of the digestive gland over total live weight.

TABLE 1.

Percentage (g/100 g) composition of six experimental diets containing various CHO levels.

	Dietary Carbohydrate Level, %					
Ingredients	**0**	**4**	**10**	**21**	**33**	**40**
Squid meal	28					28
Casein	22					22
Wheat Starch						40
Wheat flour	0	5	15	35	53.5	
Fish Meal chili (a)		30	30	30	30	
CPSP 90% (b)		10	10	5	5	
Soyabean meal		38.5	33.5	18.5		
Wheat gluten		5				
Krill paste		2	2	2	2	
Na Alginate		2.5	2.5	2.5	2.5	
Fish oil	5	3	3	3	3	5
Na_2HPO_4	5					5
KH_2PO_4	5					
Soybean lecithin		2	2	2	2	
Rovimix (c)	1	1	1	1	1	1
minerals mix (d)		1	1	1	1	
g/100 g	60	100	100	100	100	100
Calculated %						
Proteins%	39.4	51	46	36	29	39.4
Lipid%	8	8	8	8	8	8
Digestive CHO%		4	9	21	32	40
DE MJ/kg (e)	10	15	15	14	14	17

a Fish meal chili: 90% CP, 10% Fat

b CPSP90: Soluble fish protein concentrate :90% crude protein

c retino palmitate: 8M UI; cholecaciferol: 196 000 UI; alpha-tocopherol acetate: 10 000mg/kg; vitamin K: 100 mg/kg; ascorbyl polyphosphate Stay-C: 15 000 mg/kg; thiamin: 700 mg/kg; riboflavin: 2000 mg/kg, pyridoxin: 1000 mg/kg; niacin: 10 000 mg/kg; Ca-pantothenate: 5000 mg/kg; cyanocobalamine: 50 mg/kg; folic acid: 250 mg/kg; Biotin: 70 mg/kg; inositol: 30 000 mg/kg; (Roche, France).

d Disodium phosphate and monopotassium phosphate in equal amount.

e 23/35/15 Kj for protein, lipid and carbohydrate, respectively (Cousin, 1995).

Glucose and Glycogen Measurements

In the first experiment, glycogen content of the digestive gland was determined from a sample of five shrimp from each treatment, at time 0 (after 12 h fasting) and 1, 2, 3, 6 and 7 hours after feeding. Glycemia was identified when the glycogen level of the digestive gland reached its maximum level after feeding. In the second experiment glycogen content of the digestive gland was determined from six shrimp from each treatment and size population after 12-hour fasting. After weighing, the digestive gland was preserved in liquid nitrogen for further glycogen determinations.

In both experiments glycogen was extracted following the method reported by Dubois et al. (1956). Each mid gut gland was first homogenized in trichloroacetic acid (TCA, 5%) for 2 min at 16,000 rpm, and centrifuged (3000 rpm for 5 min). This procedure was done twice. One mL of TCA was pipetted into a tube and mixed with 5 volumes of 95% ethanol. The tubes were then placed in an oven at 37–40°C for 3 h. After precipitation, the tubes were centrifuged at 3000 rpm for 15 min. The precipitate containing the glycogen was dissolved by addition of 0.5 mL of boiling water and 5 mL of concentrated sulfuric acid and phenol (5%). The contents of the tubes were transferred to a colorimeter tube and read at 490 nm in a microplate spectrophotometer.

Statistical Analysis

The effect of different dietary levels of carbohydrate on the glucose content of digestive gland glycogen was analyzed separately using ANOVA. Homogeneity of variances was verified with the Cochran's test. Means for each treatment were compared using Duncan's multiple range test only after ANOVA indicated significant differences among all the dietary treatments. The arc sin transformation was used before testing the difference between percentage data (Zar 1974)

RESULTS

First Experiment

Both preprandial and during glycemia, the DGI changed in terms of CHO levels (Fig. 1). In preprandial condition, the highest DGI (4.8%) was in shrimp acclimated with 1% CHO dietary level ($P < 0.05$). The lowest DGI level (3.0%) was in shrimp fed with 33% CHO. During glycemia, the DGI increased between 22% and 57% with the lower values in shrimp fed with 33% CHO level (DGI = 3.9%) and the highest in shrimp fed with 1% CHO level (DGI = 7.3%) ($P < 0.05$). There were no statistical differences between DGI of shrimp fed with 10% and 21% CHO levels (mean value of DGI = 4.6%) (Fig. 1).

A higher preprandial digestive gland glycogen concentration was measured in shrimp acclimated at a 1% dietary CHO level (8.8 mg/g) in comparison to that obtained in the rest of CHO level (mean value of 6.3 mg/g) (Fig. 2; $P < 0.05$). A similar behavior was observed during glycemia with high glycogen concentration in shrimp fed 1% CHO levels (15.3 mg/g). A mean value of 9.7 mg/g

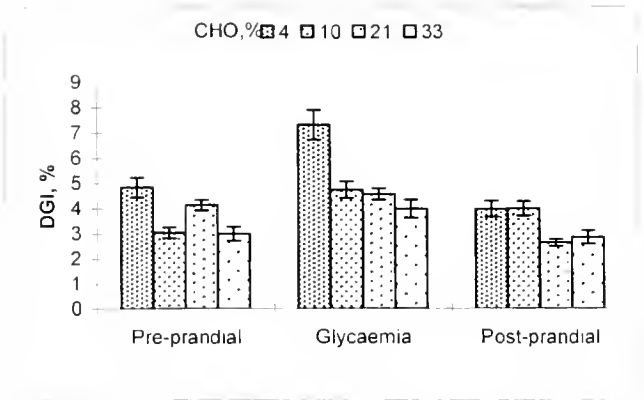


Figure 1. Wet digestive gland index (%) of *Litopenaeus stylirostris* juveniles after feeding with different carbohydrates (CHO) levels. Mean ± S.E. First Experiment.

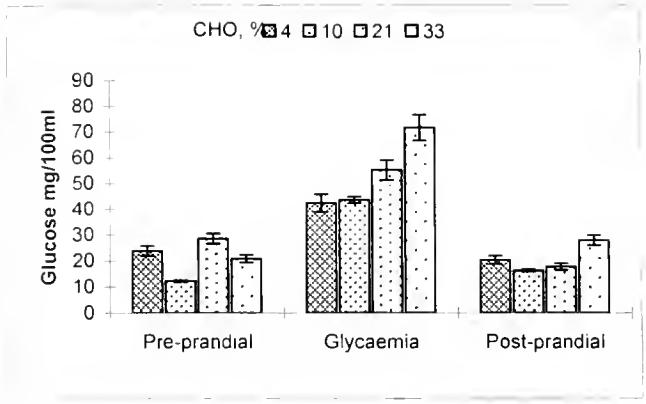


Figure 3. Glucose hemolymph levels (mg/100 ml) of *Litopenaeus stylirostris* juveniles after feeding with different carbohydrates (CHO) levels. Mean ± S.E. First Experiment.

of digestive glycogen was recorded in shrimp fed with 10%, 21%, and 33% CHO (Fig. 2).

Preprandial hemolymph levels did not show any relation to dietary CHO levels (Fig. 3). The highest values were recorded in shrimp fed 1% and 21% CHO (mean value of 25 mg/100 mL) and the lowest in shrimp fed with 10% CHO (12.3 mg/100 mL). An intermediate value (20.8 mg/100 mL) was obtained in shrimp fed with 33% CHO. After a meal, glucose hemolymph level increased between 50% and 241%, with lower values in shrimp fed with 1% and 10% CHO (mean value of 42.5 mg/100 mL) and the highest in shrimp fed with 33% CHO (71 mg/100 mL) (Fig. 3) ($P < 0.05$). An intermediate value of glucose hemolymph concentration (55 mg/100 mL) was obtained in shrimp fed with 21% CHO.

Second Experiment

The highest DGI was measured in shrimp fed without dietary CHO when shrimp were fed in an intensive feeding schedule: both juveniles and pre adults (Fig. 4). In juveniles, the highest DGI (0% CHO) was 21% higher than that observed in shrimp fed with 40% CHO ($P < 0.05$). In pre adults the highest DGI (0% CHO) was 9% higher than that measured in shrimp fed with 40% CHO ($P < 0.05$). In both diets, the DGI changed with age; juveniles were between 94% and 119% DGI higher than that observed in pre adult shrimp (Fig. 4; $P < 0.05$).

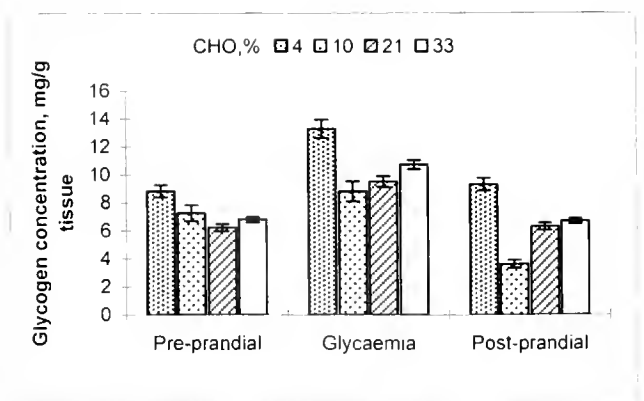
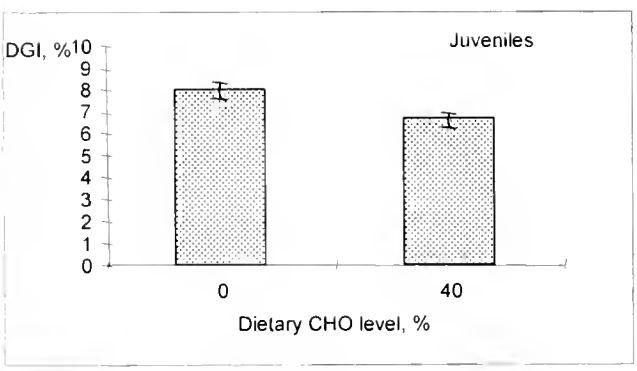


Figure 2. Digestive gland glycogen (mg/g tissue) of *Litopenaeus stylirostris* juveniles after feeding with different carbohydrates (CHO) levels. Mean ± S.E. First Experiment.

No statistical differences were observed in digestive gland glycogen of shrimp fed with high and low dietary CHO levels (Fig. 5).

DISCUSSION

The digestive gland index (DGI) of *L. stylirostris* premolt shrimp is affected by CHO level in diet. According to many workers, the digestive gland (DG) is the principal storage organ of glycogen and represents more than 80% of reserves and is the site for gluconeogenesis (Gibson & Barker 1979, Lallier & Walsh 1991, Oliveira & Da Silva 1997). The changes of weight in DG can be attributed to the glycogen storage. Part of dietary glucose will be converted to glycogen affecting the weight. Three and five



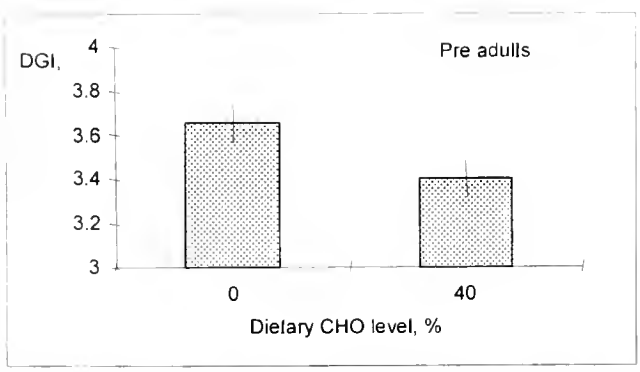


Figure 4. Wet digestive gland index (%) of *Litopenaeus stylirostris* pre adults fed with 0 and 40% dietary carbohydrates (CHO) levels. Mean ± S.E. Second Experiment.

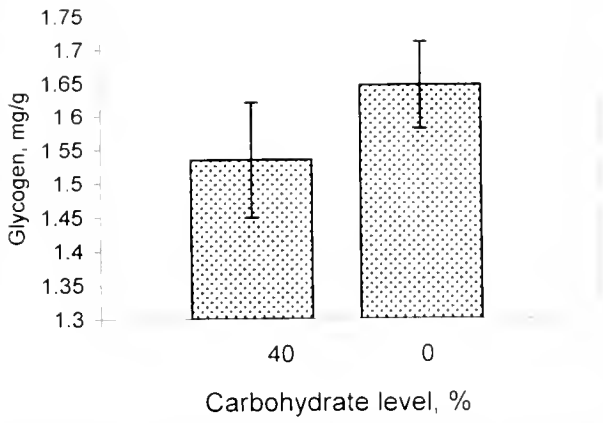


Figure 5. Digestive gland glycogen (mg/g wet tissue) of *Litopenaeus stylirostris* pre adults fed with 0 and 40% dietary carbohydrates (CHO) levels. Mean ± S.E. Second Experiment.

hours after a meal, a peak in DGI and glycogen concentration was measured in *L. vannamei* juveniles (Cousin 1995) and in *L. setiferus* adult males (Rosas et al. 1995b).

Both experiments in the present study, juveniles and pre adults showed the highest DGI and glycogen concentration were obtained when shrimp were fed with low dietary CHO level or without dietary CHO, showing that without dietary CHO the gluconeogenic pathway was induced (Fig. 1, Fig. 4). This induction means that *L. stylirostris* is well adapted to use protein as a source of metabolic energy and to make glycogen. According to Cuzon et al. (1998), *L. stylirostris* is omnivorous-carnivorous, justifying its adaptation to process protein-rich diets.

During the molting process (stages Do to D1‴), crustaceans not only accumulate glycogen but also triglycerides and proteins in the DG in preparation for chitin synthesis (Santos & Keller 1993). During this time, the hexokinase activity is increased to accumulate glycogen, reducing the pentose pathway in preparation of chitin synthesis and the demands for energy (1.4 kJ representing about 25% of the energy accumulated along the intermoult cycle), which is provided through the glycolysis pathway. The highest levels of DGI and DG glycogen observed in shrimp fed without CHO reflect the amount of glycogen that the shrimp needs to support both the chitin synthesis and the production of metabolic glucose. This has been observed in other shrimp species in stage C of molt (Rosas et al. 2000, Rosas et al. 2001a, Rosas et al. 2001b). In contrast, in shrimp fed with CHO rich diets a high gluconeogenesis is not necessary because shrimp have enough CHO from the diet to satisfy the chitin synthesis and glucose for energetic demands producing, in consequence, the lowest levels of the DGI and DG glycogen.

In a recent paper (Rosas et al. 2001a), we demonstrated that *L. vannamei* can convert protein to glycogen by a gluconeogenic pathway when fed with 1% dietary CHO. In that study we measured a high phosphoenolpyruvate carboxykinase (PEPCK) activity indicating that the gluconeogenic pathway is induced to produce glycogen and glucose. This activation allowed maintenance of a relatively high circulating glucose of 0.34 mg/mL in hemolymph in comparison to that observed in shrimp fed with 30% dietary CHO (0.45 mg/mL). A similar pattern has been observed in *Chasmagnatus granulata* crabs (Oliveira & Da Silva 1997) in which the production of glucose from ^{14}C alanine was demonstrated.

This type of strategy is not exclusive to shrimp. In a carnivorous fish such as cod, the hepatosomatic index (HIS) followed a similar pattern as with *L. stylirostris* with a HSI of 6.5% when fish were fed without CHO and 5.3% when fed with 40% starch. These results suggest that cod are well adapted to obtain CHO from protein, knowing this fish in the wild does not have dietary CHO (Hemre et al. 1990).

The present results can be related to the adaptation of shrimp to respond to the protein and carbohydrate variations in wild also. Donaldson (1976) demonstrated that, depending on the season of the year; proteins are the most abundant molecules in benthic ecosystems ranging between 46% to 72% in comparison to 1% to 2.5% of CHO. That observation suggest that wild shrimp are obligated to adapt to synthesize CHO according to environmental protein fluctuations, adjusting their metabolism to be dependent on the abundance of proteins more than CHO.

During glycemia, the highest DGI and glycogen levels were measured in *L. stylirostris* fed without dietary CHO, both in an intensive feeding schedule and after a single meal. Between juveniles (Fig. 1, Fig. 4), the highest DGI was observed in shrimp fed intensively, indicating that in this type of feeding regimen the gluconeogenesis pathway can be forced to produce more glycogen

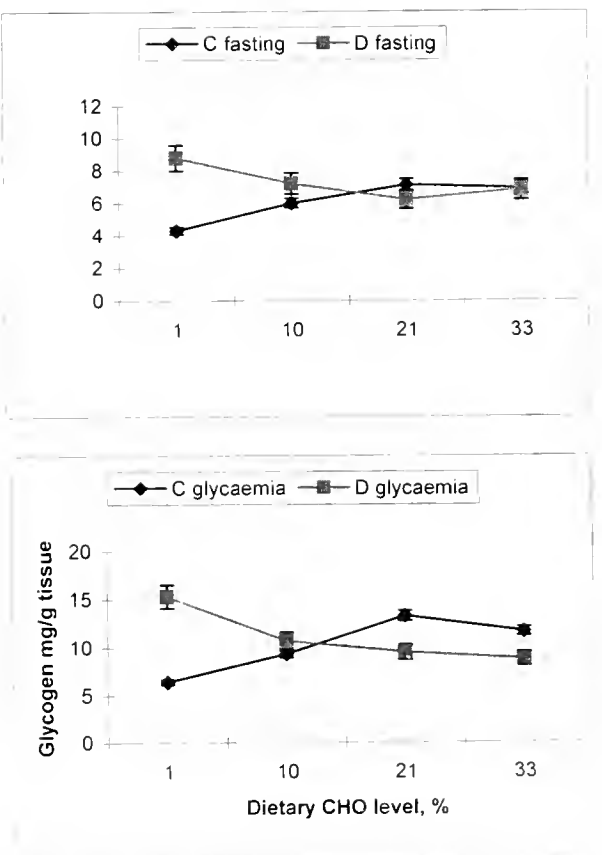


Figure 6. Digestive gland glycogen (mg/g wet tissue) of *Litopenaeus stylirostris* fed with different CHO levels and with shrimp 12 h fasting and during glycaemia. Data for stage C from Rosas et al., (2000). Mean ± S.E.

than that observed only after a meal. In the intensive feeding trial, shrimp were fed every hour starting from 0800 to 1630, which represented around seven meals a day. This rhythm of feeding procured a constant high blood glucose level and the highest glycogen level in the DG. Feeding has been shown to decrease hemolymph hyperglycemic hormone (CHH) levels activating the glycogen synthesis, resulting in increase of glycogen content in different tissues (Santos & Keller 1993). If high glucose remains for a long time in the blood after feeding, the control of CHH could be reduced maintaining the glycogen synthesis for a long time and evidencing the poor regulation of CHO metabolism by shrimp. Similar results were shown by Cousin (1995) in a previous study in which he observed the postprandial effect of different starches in *L. vannamei* juveniles. All these results suggest that the gluconeogenesis process is fast and independent of the molt stage. In previous work, we observed in *L. stylirostris* fed without CHO that the peak of glycogen after a single meal is between two to three hours (Rosas et al. 2000). In this present study made with shrimp in stage D, glycemia was reached two hours after feeding when shrimp were fed after 12 hours fasting. These results could indicate that the molting stage changes the intensity of the process but not its rate. The use of labeled CHO in diets could help confirm this hypothesis.

The age of shrimp does not affect the intensity of gluconeogenesis in shrimp fed without CHO. As in juveniles, pre adults had the highest DGI and DG glycogen levels when they were fed without CHO. The shrimp culture industry had demonstrated that shrimp broodstock are more carnivorous than juveniles, because at that age the shrimp needs more protein and lipids for sexual maturation (Bray & Lawrence 1992). For this reason the gluconeogenesis in the adult phase could be more important than in juveniles.

Comparing the results obtained in the present paper with those for *L. stylirostris* juveniles and published in Rosas et al. (2000), it is possible to observe that both in 12 hour fasting and during glycemia, the glycogen concentration in stage C shrimp was the inverse of that measured in stage D shrimp (Fig. 6). These results show that the intensity of gluconeogenesis pathway changes with the molting stage, being more important in the pre molt stage (D) than during the inter molt stage (C). In the same paper, a significant induction of α-amylase and α-glucosidase activities related to an increase in dietary CHO levels were measured in *L. stylirostris* juveniles indicating that in stage C the dietary CHO affected directly the CHO metabolism.

During growth, the molting process requires the shrimp to make dramatic physiological, behavioral, and metabolic changes to satisfy energetic and structural demands (Bauer 1996, Carvalho & Phan 1998, Charmantier et al. 1994). To satisfy energetic demands, *L. stylirostris*, as other shrimp species, is well adapted to use protein independently of the dietary CHO. The DG changes observed in the present study confirm that assumption and the limited ability of this shrimp species to use dietary CHO, mainly when the glycogen demands are enhanced by the molting stage.

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THE CONTENT OF ASCORBIC ACID AND TOCOPHEROL IN THE TISSUES AND EGGS OF WILD *MACROBRACHIUM ROSENBERGII* DURING MATURATION

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ABSTRACT Variations in the concentrations of ascorbic acid (AA) and tocopherols in association with the gonadal development of the freshwater prawn *Macrobrachium rosenbergii* were investigated in females captured in the Mae Klong River, Thailand. Mean ovarian AA levels ranged from 210 to 540 μg/g dry weight (dw) and were at least 11-fold higher than midgut gland (MG) levels. Variations in ovarian AA levels are believed to be related to the biosynthesis of steroid hormones, the formation of collagen, and the deposition of egg yolk compounds. α-Tocopherol (α-T) was the predominant form of vitamin E in prawn tissues and eggs. The level of α-T in the MG was constant, whereas in the ovaries, it ranged from 143 to 425 μg/g dw. The incorporation of α-T into the ovary was highly correlated ($r^2 = 0.87$) to ovarian lipid levels, which probably reflects the role of this vitamin as a major antioxidant agent. The present results provide further evidence of the essentiality of these vitamins in crustacean reproduction.

KEY WORDS: ascorbic acid, tocopherols, *Macrobrachium rosenbergii*, wild, reproduction, nutrition

INTRODUCTION

Although in the last two decades much progress has been achieved in the understanding of vitamin metabolism in crustaceans (Conklin 1997), knowledge concerning the role of vitamins in crustacean reproduction is still limited (Harrison 1990, Harrison 1997). As a result, most information on vitamin functions and requirements are adopted from literature on fish and other vertebrates, rather than being derived from studies with crustaceans (Harrison 1990).

Vitamin E (tocopherol) and vitamin C (ascorbic acid [AA]) are considered essential dietary components for crustaceans (Conklin 1997). The biological activity of vitamin E is widely accepted to be at least partially related to its antioxidant properties, as it reacts rapidly with organic free radicals that may damage membrane-bound polyunsaturated fatty acids (PUFA) (Burton & Trabor 1990). Vitamin E and C are known to act synergistically, with vitamin E reacting with lipid peroxy radicals donating a hydrogen atom and forming a vitamin E radical, which is then regenerated by AA (Packer et al. 1979). The importance of vitamin E for fish reproduction has long been recognised (Watanabe & Takashima 1977, Watanabe et al. 1985), but has only been recently demonstrated in crustaceans (Cahu et al. 1991, Alava et al. 1993b, Cahu et al. 1995).

Aside from its role in the recycling of vitamin E, AA also participates in the enzymatic processes involved in the formation of collagen (Barnes 1975; Hunter et al. 1979) and in the biosynthesis of steroid hormones (Hilton et al. 1979; Seymour 1981). Although the need for AA in diets for fish broodstock has been well established (Watanabe & Takashima 1977, Sandnes et al. 1984, Soliman et al. 1986, Waagbo et al. 1989, Dabrowski 1991, Blom & Dabrowski 1995), the essentiality of AA in crustacean reproduction was initially inferred from a study evidencing its variation in the ovary of *Palaemon serratus* Pennant (Guary et al. 1975). More recent studies on penaeid shrimps have confirmed its importance in crustacean reproduction (Alava et al. 1993a, Alava et al. 1993b, Cahu et al. 1995).

Under rearing conditions, feed regimes for the freshwater prawn *Macrobrachium rosenbergii* (de Man) range from fresh food to formulated feed, and thus vitamin rations may vary considerably. As no information is available on the status of vitamin E and C in adult prawn tissues during maturation, it is not possible yet to establish criteria for evaluating the ovarian status of these vitamins or to recommend modifications in the dietary vitamin content so as to optimize broodstock performance and offspring quality. With this in mind, this paper aims to present baseline data on the concentrations of AA and tocopherols in the midgut gland, ovary, and eggs of wild *M. rosenbergii* throughout sexual maturation, and it discusses the possible roles that these vitamins may have in the reproduction of this species.

MATERIAL AND METHODS

Live mature *M. rosenbergii* females were obtained from fishermen in the Mae Klong River, Amphur Muang, Province of Samut Songkhram, Thailand. Captures were made in single collections on July and September 1998. After capture, female prawns were grouped in five stages of gonadal development, according to the size, colour, and aspect of the ovary (Chang & Shih 1995), i.e., (I) no ovarian tissue is visible, which is characteristic of both nondeveloped and spent females; (II) the ovary has a small yellow-colored spot near the posterior part of the carapace; (III) the ovarian tissue turns orange and is visible from the posterior part of the carapace to the area just in front of the epigastric tooth; (IV) the ovarian tissues have grown and extended to the area of the epigastric tooth; and (V) the ovarian tissues have extended to the anterior part of the carapace. Females in all five stages of gonadal development were sampled on July and September.

Females were then blotted dry and were individually measured (total length from the tip of the rostrum to the end of the telson) and weighed (to the nearest 0.1 g). The ovary and midgut gland were quickly dissected, weighed, and immediately frozen at −20°C. Grayish, eyed-eggs were also sampled and conserved in a

TABLE 1.

Weight, total length, ovary weight, and midgut gland weight of wild *M. rosenbergii* females at different stages of gonadal development. Each value is the mean of four separate prawn samples analyzed individually, except for stages I and II where tissues of three prawns were pooled. Within rows, values with different superscript letters indicate significant differences ($P < 0.05$).

	Stage of Gonadal Development				
	I	II	III	IV	V
Prawn weight (g)	33.4 ± 12.4	34.3 ± 10.1	41.4 ± 12.5	36.7 ± 11.6	38.8 ± 10.2
Prawn length (cm)	14.4 ± 1.1	14.7 ± 1.4	15.2 ± 1.9	15.4 ± 1.7	15.5 ± 1.4
Midgut gland weight (g)	1.37 ± 0.50	1.38 ± 0.39	1.79 ± 0.54	1.53 ± 0.48	1.73 ± 0.45
Ovary weight (g)	0.17 ± 0.06^{c}	0.53 ± 0.15^{c}	1.16 ± 0.35^{bc}	1.62 ± 0.51^{ab}	2.33 ± 0.60^{a}

similar manner. The gonado-somatic index (GSI) and midgut somatic index (MSI) were calculated as the percentage of gonad and midgut gland to total body weight, respectively. All samples were conditioned in a Styrofoam box with dry ice and were transported by air to Belgium. Samples for AA and tocopherol analysis were then maintained at –80°C and –20°C until analysis, respectively. The content of AA of the samples was determined according to Nelis et al. (1997), while α-tocopherol (α-T), γ-tocopherol (γ-T), and δ-tocopherol (δ-T) levels were estimated following Huo et al. (1999). Tissue samples from the same wild *M. rosenbergii* females were utilized in a parallel study describing the variation of total lipids, lipid classes, and fatty acids (Cavalli et al. 2001).

Statistical analysis of the data was undertaken with one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test. An alpha level of 0.05 was used to identify significant differences. Correlations were determined using linear regression analysis. A minimum of three replicates for each tissue and eggs were analyzed. When tissues of one individual were insufficient for analysis, tissues were pooled from 2 to 4 individuals. This was especially true for the early stages of gonadal development. Results are presented as means ± SD.

RESULTS

Prawn weight, total length, and midgut gland weight presented no significant differences between the various stages of maturation, but ovarian weight tended to increase, especially after stage III (Table 1). Changes in MSI and GSI during maturation are shown in Figure 1. MSI was constant irrespective of maturation stage, whereas GSI presented a significant increase from stages I to V.

Figure 2 summarizes the data on the concentrations of AA in the midgut gland (MG) and ovary throughout maturation. The content of AA in the MG was constant in the early stages of maturation, but decreased significantly from stage III (mean of 24.9 μg/g dw) to stage V (12.8 μg/g dw). In the ovary, the AA concentrations were stable at around 540 μg/g dw at stages I and II, but declined significantly between stages II to IV. At stage V, ovarian AA levels increased significantly to 370 μg/g dw. Within the same stage of gonadal development, mean AA levels were 11- to 29-fold higher in the ovary than in the MG. Eggs contained 128.2 ± 28.3 μg AA/g dw (Table 3).

The main form of tocopherol present in the MG, ovary, and eggs was α-T (Tables 2 and 3). In the MG, there were no significant differences in tocopherol content, as the variations were large. The content of α-T in the ovary increased significantly during the initial stages of gonadal development (from stages I to III), and decreased afterwards. The levels of γ-T were not significantly different at any given stage of maturation, and δ-T levels were below the detection limit during the initial stages of maturation. The mean content of α- and γ-T in the eggs was 324.7 and 22.8 μg/g dw, respectively (Table 3). No δ-T was detected in the eggs.

Linear regression analysis of the total lipid content in the ovary (data from Cavalli et al. 2001) against the concentration of α-T produced a correlation coefficient (r^2) equal to 0.87 (Fig. 3). Correlations between ovarian total lipids and γ- and δ-T levels were not significant.

DISCUSSION

Although only a limited number of studies report AA levels in crustaceans, it is well documented that tissue levels vary with season, ontogenetic development, dietary intake, moulting, and reproductive cycles (Guary et al. 1975, Magarelli & Colvin 1978, Coglianese & Neff 1981, Merchie et al. 1995). In fish, several

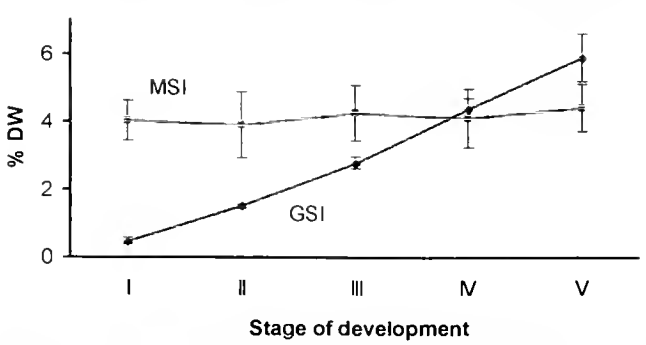

Figure 1. Changes in GSI and MSI of wild *M. rosenbergii* females at different stages of gonadal development.

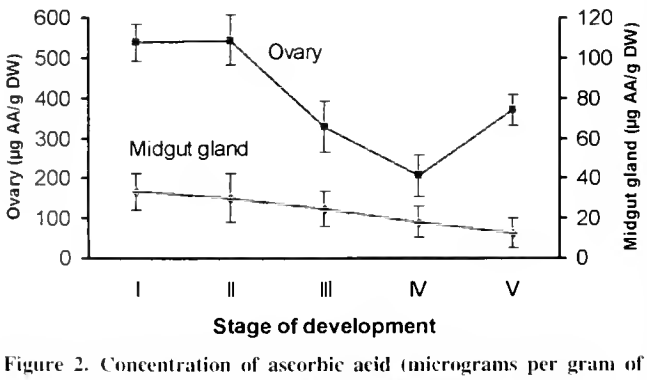

Figure 2. Concentration of ascorbic acid (micrograms per gram of dw) in the midgut gland and ovary of wild *M. rosenbergii* females according to the stage of gonadal development.

TABLE 2.

Concentration of tocopherols (micrograms per gram of dry weight) in the midgut gland and ovary according to the stage of gonadal development of wild *M. rosenbergii* females. Each value is the mean of four separate prawn samples analyzed individually, except for stages I and II where tissues of three prawns were pooled. Within rows, values with different superscript letters indicate significant differences ($P < 0.05$).

	Stage of Gonadal Development				
	I	II	III	IV	V
Midgut gland					
α-Tocopherol	153.7 ± 72.2	174.9 ± 70.3	136.3 ± 92.8	31.6 ± 12.3	102.3 ± 26.5
γ-Tocopherol	21.1 ± 18.1	13.0 ± 11.3	9.8 ± 1.6	2.7 ± 0.9	9.7 ± 1.6
δ-Tocopherol	0.5 ± 0.7	0.3 ± 0.3	n.d.	n.d.	1.0 ± 0.5
Ovary					
α-Tocopherol	142.6 ± 13.0^{c}	334.0 ± 79.9^{ab}	425.0 ± 67.7^{a}	260.3 ± 33.5^{bc}	279.1 ± 37.1^{bc}
γ-Tocopherol	11.0 ± 1.9	22.1 ± 2.1	13.1 ± 0.8	16.3 ± 1.5	17.9 ± 7.1
δ-Tocopherol	n.d.	n.d.	n.d.	n.d.	0.6 ± 0.4

n.d. = not detected

authors (Seymour 1981, Sandnes & Braekman 1981, Dabrowski 1991) have demonstrated that the levels of AA in the ovaries change during the reproductive cycle. Sandnes and Braekman (1981) showed a rise in ovarian AA concentration during ovarian growth followed by a decrease in the final stages prior to spawning, and they discussed whether this variation could be related with sex steroid synthesis. Guary et al. (1975) also postulated that the decrease in AA levels in the maturing ovary of *P. serratus* could be connected to steroidogenesis. In the present study, the decline of AA levels in the ovary between stages II to IV coincides with an active phase of ecdysteroid hormone accumulation in the maturing ovaries of *M. rosenbergii* (Wilder et al. 1991). Furthermore, significant levels of cholesterol, the chief precursor of steroid hormones (Kanazawa & Teshima 1971), were present in the ovary of *M. rosenbergii* throughout maturation (Cavalli et al. 2001). These findings reflect a possible demand for AA by the hydroxylating reactions needed for steroidogenesis in the ovarian follicle cells, and they agree with results revealing the possibility of endogenous production of steroid hormones in crustaceans (Kanazawa & Teshima 1971, Shih & Liao 1998).

The decrease in the ovarian AA content between stages II and IV could also be linked to the biosynthesis of collagen, as the hydroxylation reaction necessary for the synthesis of this fibrous protein requires the presence of AA at adequate levels (Hunter et al. 1979). In this respect, the concentration of total ovarian protein has been shown to increase linearly along with GSI in *M. rosenbergii* (Lee & Chang 1997).

Guary et al. (1975) suggested that the formation of egg yolk compounds, such as polysaccharides and glycogen, also require considerable amounts of AA and therefore could be an additional cause for the decrease in ovarian AA levels, particularly at the final stages of gonadal development. Although the possibility that these metabolic processes consume some AA cannot be ruled out, the observation that the levels of AA in the ovary of *M. rosenbergii* increased from stage IV to V suggests that the deposition of AA into the ovary at the final stages of maturation occurs at a much higher rate than its catabolism.

The raise in ovarian AA content in the final stages of maturation may be related to an increased requirement in the egg at a later stage of life (Hilton et al. 1979). Indeed, it was found in various fishes (Hilton et al. 1979, Dabrowski 1991, Blom & Dabrowski 1995) and crustaceans (Guary et al. 1975, Coglianese & Neff 1981, Alava et al. 1993a) that AA levels in the ovaries are usually higher than in other tissues. Similarly, in the present study, the ovary of *M. rosenbergii* contained significantly more AA than the MG. Sandnes et al. (1984) and Soliman et al. (1986) confirmed that an important share of the broodstock dietary AA intake is transferred to the oocytes where it is stored for use during embryogenesis and larval development. This clearly indicates a preferential transfer of reserve AA to the embryos, which is particularly important in *M. rosenbergii* since the embryos and early larvae are totally dependent on the yolk reserves for normal organogenesis and physiological functioning (Harrison 1990). Several authors have shown that the viability of fish eggs (Sandnes et al. 1984, Soliman et al. 1986, Waagbo et al. 1989) and shrimp eggs (Cahu et al. 1995) was directly related to their AA content. The improvement in egg viability with increased AA levels was attributed to the protection of membrane-bound lipids against oxidation and by the action of this vitamin in the synthesis of stable forms of collagen (Cahu et al. 1995), as suggested for fish (Waagbo et al. 1989).

In an earlier study, De Caluwé et al. (1995) collected *M. rosenbergii* eggs 2 days after fertilization and found that mean AA levels in the eggs varied between 210 and 382 μg/g dw. The upper limit of this range agrees well with the ovarian AA content at the final stages of maturation in the present study (around 370 μg/g dw), but is relatively higher than the 128 μg AA/g dw found in the eggs. However, as the eggs sampled here were "eyed" and gray colored, and were thus at the final stages of embryonic development (New & Singholka 1982), this suggests that the developing

TABLE 3.

Concentration of ascorbic acid and tocopherols in the eggs of wild *M. rosenbergii* females.

	Mean ± SD (μg/g dry weight)
Ascorbic acid	128.2 ± 28.3
α-Tocopherol	324.7 ± 77.3
γ-Tocopherol	22.8 ± 10.4
δ-Tocopherol	n.d.

n.d. = not detected

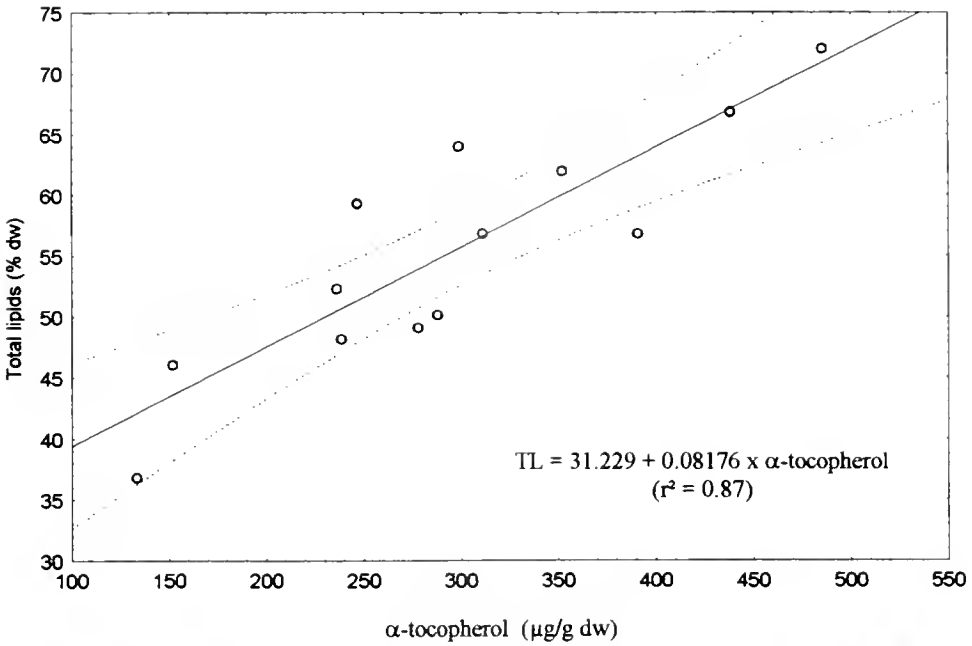

Figure 3. Linear regression analysis between the total lipids (TL) and the contents of α-T in the ovary of wild *M. rosenbergii* females.

embryos possibly consumed AA. Moreover, the fact that newly hatched, nonfeeding larvae of *M. rosenbergii* contained from 149 to 265 μg AA/g dw (Cavalli et al., 2000) further indicates that AA was indeed consumed by the developing embryo. This possibility is also supported by the results of Sato et al. (1987) who demonstrated a continuous decrease in AA content of rainbow trout eggs during embryonic development. Conversely, Guary and Guary (1975) reported that the eggs of *P. serratus* and *Cancer pagurus* (L.) seemed able to synthesize AA during the early stages of embryonic development, and hence, AA contents after spawning were found to be similar to those just before hatching. However, from a metabolic standpoint, it seems unlikely that a female shrimp would accumulate considerable amounts of AA into its gonad (Guary et al., 1975) if the eggs were able to biosynthesize it during the early stages of embryonic development. Therefore, it remains to be confirmed whether the biosynthesis of AA occurs during the embryonic development of crustaceans.

Watanabe et al. (1985) reported that vitamin E, together with lipids, was easily incorporated into red sea bream eggs. According to the present results, the incorporation of α-T into the ovary of *M. rosenbergii* was highly correlated to ovarian lipid levels. This finding is in agreement with the antioxidative role of this fat-soluble vitamin, which requires its close association with lipids, particularly membrane-bound PUFA. Therefore, it is possible that to fulfill its vitamin E requirements, reproductive *M. rosenbergii* females would depend more on the dietary intake than on body reserves, as was hypothesized for lipids (Cavalli et al. 2001). Nevertheless, the contribution of MG and muscle reserves may also be of some importance. Data from the studies of Castillo et al. (1989) and Alava et al. (1993b) indicate that α-T might have been transferred from these tissues to the eggs of *P. indicus* and *P. japonicus*, respectively. It is still unclear whether this is also true for *M. rosenbergii*.

De Caluwé et al. (1995) found that *M. rosenbergii* eggs had from 711 to 1,287 μg α-T/g dw. These concentrations are much higher than those found in the present study, and they suggest a comparatively lower dietary intake of vitamin E under natural conditions. In fact, the rate of incorporation of vitamin E into the eggs of *P. indicus* and *M. rosenbergii* was shown to increase with dietary levels of α-TA (Cahu et al. 1991, De Caluwé et al. 1995). *M. rosenbergii* females fed a diet containing 223 μg α-TA/g dw produced eggs with an average of 711μg α-T/g dw, while the content of α-T in the eggs almost doubled to 1,287 μg/g dw when dietary α-TA levels were increased to 2,025 μg/g dw.

In summary, the present study provides further evidence of the importance of AA and tocopherols in the reproduction of crustaceans, and consequently suggests that feeding a diet deficient in either vitamin C or E could virtually impair broodstock performance and offspring viability. More research is necessary to determine optimal dietary levels for crustacean broodstock.

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GROWTH AND GAMETOGENIC CYCLE OF THE CRESTED OYSTER, *OSTREA EQUESTRIS* (SAY, 1834), IN COASTAL GEORGIA

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ABSTRACT Crested oysters, *Ostrea equestris* (Say, 1834), were collected from the mouth of the Savannah River on December 2, 1998 and transferred to pearl nets suspended from the main dock at the Skidaway Institute of Oceanography on the Skidaway River. To determine the reproductive cycle and growth of oysters, 50 oysters were collected monthly, measured for shell length, and a gonadal sample was taken for histological analysis. Oysters grew from a mean size of 18.8 mm in January 1999 to 28 mm by November 1999—a rate of 0.9 mm per month. Of 574 oysters sectioned for histological examination, males dominated the population (49.5%), with only 3.5% being females, 4.5% were indeterminate and 42.5% being hermaphrodites. Crested oysters were reproductively active year around in Georgia with spawning and spent oysters occurring in most months. In males, spawning and spent stages ranged from 23.7% of the oysters in January to 86.4% in August, while females in these stages ranged from 10.7% in February to 77.8% in August. Brooding larvae were found in the histological samples between April and October. Spawning of crested oysters in Georgia occurs year round with major spawning starting in February and continuing into fall.

KEY WORDS: crested oyster, *Ostrea equestris*, growth, gametogenesis, hermaphroditism

INTRODUCTION

The crested oyster, *Ostrea equestris* (Say 1834), occurs from North Carolina to both coasts of Florida, throughout the Gulf of Mexico and to the West Indies (Abbott 1974). Externally, the crested oyster appears similar to the eastern oyster, *Crassostrea virginica* (Gmelin 1791), but can be distinguished from it by a single row of denticles along the inner lateral margin of the upper valve with corresponding depressions on the lower valve (Galtsoff & Merrill 1962). The crested oyster is a high saline organism occurring in estuarine areas greater than 20 ppt to oceanic conditions (Galtsoff & Merrill 1962). It grows to 72 mm in length in the Gulf of Mexico (Gunter 1951) and to 82 mm on the Atlantic coast (Galtsoff & Merrill 1962). Crested oysters do not form beds like *C. virginica* (Galtsoff & Merrill 1962). *O. equestris* will replace *C. virginica* on beds when salinity increases to 30 ppt (Parker 1960). Hoese (1960) observed in Texas at the end of a drought, an *O. equestris - Brachidontes exustus* community was replaced by a *C. virginica - B. recurvus* community.

Although there have been several reports published on the biology of *O. equestris* from the Gulf of Mexico region (Gunter 1951, Menzel 1955, Parker 1960, Hoese 1960) and one distributional study on the Atlantic coast (Galtsoff & Merrill 1962), studies on life history traits for Atlantic coast populations are absent. This study describes the growth and gametogenic cycle of the crested oyster in the coastal waters of Georgia.

MATERIALS AND METHODS

Crested oysters were collected by the R/V Georgia Bulldog while trawling with a 3 m conch try net at the mouth of the Savannah River (32°02′42″ X 80°50′21″) 1998 December 2. The sampling depth was 6 m and the sediment was dominated by shelly material. Surface salinity was 24 ppt. Oysters attached to relic *C. virginica* shells were returned to the laboratory where 50 non-randomly selected oysters were measured for shell length (i.e., hinge to lip) to the nearest 0.5 mm with Vernier calipers. This initial sample was non-random because large-sized oysters were selected for ease of identification. After measuring each oyster, the internal lateral margin of each oyster shell was examined to ensure identification and a 1cm^2 gonadal sample was removed and preserved in Davidson's solution. Remaining oysters were placed into 12-mm mesh pearl nets and suspended from the main dock at the Skidaway Institute of Oceanography on the Skidaway River.

Each month 50 oysters were randomly gathered, measured for shell length, and a gonadal sample (ca. 1 cm^2) was dissected from each oyster. These oysters were selected by gathering two *C. virginica* shells from the pearl nets with attached crested oysters. Tissues were processed according to procedures outlined in Howard and Smith (1983). Prepared slides of gonadal tissue were examined with a Zeiss Standard 20 microscope (×20), sexed and assigned to a developmental stage as described by Pouvreau et al. (2000). A staging criteria of 0 to 6 was employed for early active (1), activity developing (2), near ripe (3), ripe (4), partially spawned (5), spent (6), and inactive (0).

Water temperatures and river salinity from the Skidaway River were taken from the Marine Extension Service's dock which is adjacent to the Skidaway Institute of Oceanography's dock at 0800 h Monday through Friday.

RESULTS

Monthly mean water temperature and salinity values for the Skidaway River are given in Figure 1. Temperatures ranged from 12.2°C in January 1999 to 30.9°C in August 1999, while river salinity ranged from 23.4 ppt in July 1999 to 29.9 ppt in December 1999. River salinity values were abnormally high especially during spring due to prolonged drought conditions in the southeast and Georgia.

Crested oyster mean size from December 1998 to November 1999 is given in Figure 2. The mean size of oysters in December 1998 (22 mm) was artificially high because larger-sized animals were selected to ensure the correct identification of the animals at the start of the experiment. The January 1999 size (18.8 mm) represents a random mean size. Thus, oysters grew from 18.8 mm in January 1999 to a mean size of 27.8 mm by November 1999—a rate of 0.9 mm per month.

A total of 574 oysters were sexed from December 1998 to November 1999. Indeterminate oysters occurred in every month except March and April and accounted for 26 individuals (4.5%).

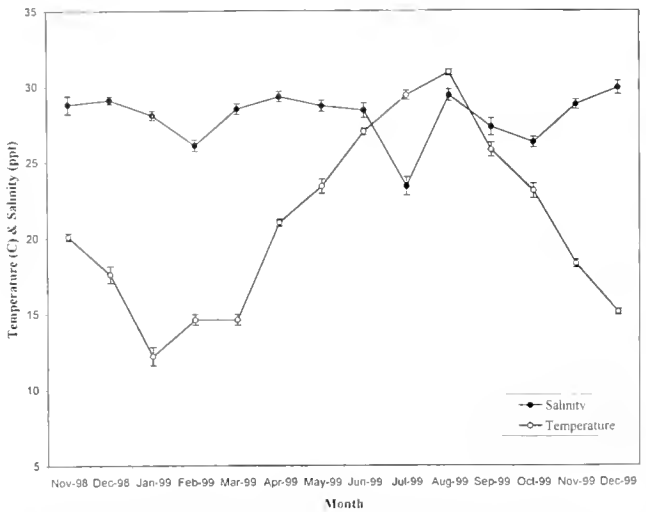

Figure 1. The mean monthly water temperature and salinity of the Skidaway River, Georgia from November 1998 to November 1999 (vertical bars indicate ± 1 standard error from the mean).

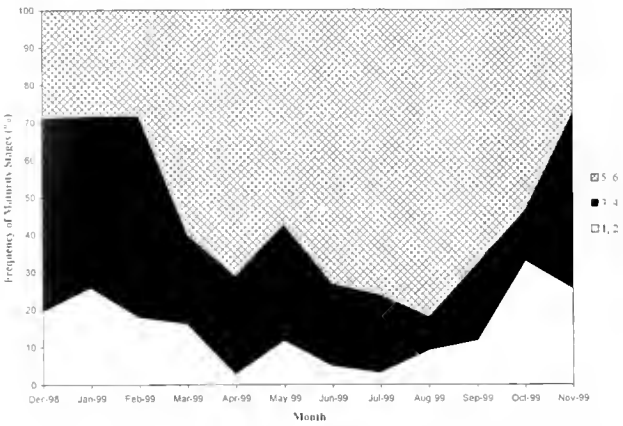

Figure 3. The reproductive phases (early active = 1, activity developing = 2, near ripe = 3, ripe = 4, partially spawned = 5, and spent = 6) of female and male crested oysters, *Ostrea equestris*, from the coastal waters of Georgia between December 1998 and November 1999.

Male individuals occurred every month and totaled 284 individuals (49.5%), while only 20 female oysters (3.5%) occurred. There were no female oysters identified in the December 1998, March 1999, April 1999 and September 1999 samples. Two hundred forty four oysters (42.5%) were hermaphroditic. Hermaphrodites occurred in each month sampled. Brooding larvae in the samples were observed from April to October.

Crested oysters in Georgia were reproductively active year around (Fig. 3). Near ripe and spawning ripe female and male oysters were found in all months. Female and male spawning and spent oysters occurred year-round with these stages dominating from March through December. Male spawning and spent stages ranged from 23.7% of the oysters in January to 86.4% in August, while in females these stages ranged from 10.7% in February to 77.8% in August. Brooding larvae were found in 3.1% of the histological samples occurring in each month from April to October. Spawning appears to be a prolonged event in Georgia starting in February and continuing into fall.

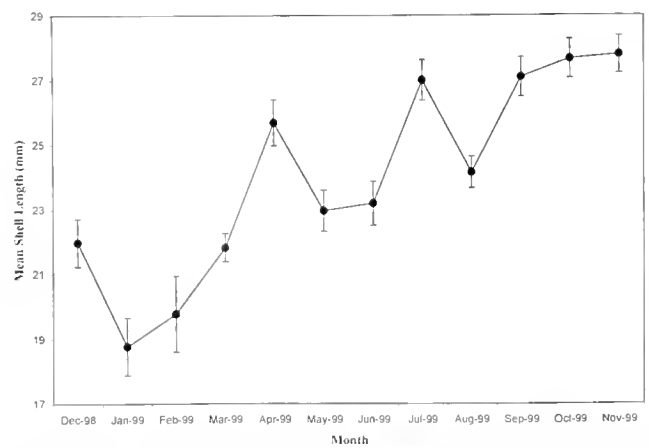

Figure 2. The mean shell length (mm ± standard error) of crested oysters from pearl nets suspended in the Skidaway River, Georgia from December 1998 to November 1999.

DISCUSSION

Within the Ostreidae family, oysters of the genus *Ostrea* are brooding species, whereas, members of the *Crassostrea* genus release their gametes into the water column (Galtsoff 1964). Brooded larvae have been documented in *Ostrea equestris* by Gutsell (1926) and Menzel (1955), and were noted in the present study from histological samples taken between April and October.

Water temperature is a controlling factor in regulating spawning and gametogenesis in marine bivalves (Giese 1959, Sastry 1975). In general spawning periods and gametogensis in marine bivalves start earlier and last longer in southern geographical areas than in northern ones (Eversole 1989, Thompson et al. 1996). *Ostrea equestris* in the subtropical waters of Georgia are reproductively active year around (Fig. 3). Although Menzel (1955) did not follow gametogensis of *O. equestris* on an annual basis, he showed that they continually recruited from the first of May until the first of November indicating prolonged spawning seasoning in Louisiana. Other warm water *Ostrea* species also have long spawning periods and periods of no reproductive inactivity. In Costa Rica, *O. iridescens* was observed spawning 9 out of 14 months, with no reproductive resting stages observed. In New Zealand, *Tiostrea* (=*Ostrea*) *chilensis* spawns year around with peak spawning in late summer-autumn and brood larvae found from July to January (Jeffs 1998, Jeffs & Hickman 2000). In colder waters, *O. chilensis* has one spawning period per year in spring to early summer with brooding lasting only 7 to 8 weeks (Chaparro & Thompson 1998). Likewise, in Argentina *O. puelchana* spawns from December to February; however, no resting stages of reproductive activity were observed with rapid proliferation of ovocytes occurring in March-April (Castro & Mattio 1987). In California *O. lurida* is reproductively active year around, but spawns only during seven months with peak spawning in June and July (Coe 1932a). Spawning in *O. lurida* ceases when water temperatures fall below 16°C (Coe 1932b). In Ireland, *O. edulis* spawns from May to September with minimum reproductive activity from October to February (Wilson & Simons 1985).

Ostrea equestris is a protandric species as are other species of *Ostrea*. Menzel (1955) followed the growth and reproductive cycle of crested oysters from set and observed that 74.4% of the oysters were males and 10% females. Of the remaining oysters, 13% were

indeterminate and only 2.6% were hermaphroditic. In our study, males also dominated (49.5%); however, the percentage of hermaphroditic individuals (42.5%) was much greater than that observed in crested oysters from Louisiana. The crested oysters from Georgia were well beyond the set stage when collected from the Savannah River. Since the crested oyster is protandric as suggested by Menzel (1955), then this could explain the difference in the percent hermaphroditism between populations. The oysters from Louisiana were younger and developing first as males, thus the 74% males, before changing to females or hermaphrodites at a later age or size. Oysters gathered in December 1998 in Georgia already ranged in size from 8.7 mm to 34 mm in shell length, and had probably already past through the initial sex change stage from males to females. In oyster populations, the closer the proximity of one oyster to another, the higher the proportion of males in that population (Burkenroad 1931, Smith 1949, Menzel 1951, Buroker 1983). In the present study, crested oysters were held close together in suspended pearl nets, which may explain the much skewed male to female ratio observed (1:0.07).

Oysters of the genus *Ostrea* have greater occurrences of hermaphrodites than species of *Crassostrea* (Table 1). Galtsoff (1964) stated that oviparous species of oysters (*Crassostrea*) are not usually hermaphroditic, while larviparous species of oysters (*Ostrea*) as a rule change in sexuality (i.e., from an initial male phase followed by alternating female and male phases). *Ostrea edulis* has long been recognized as a protandric species; spat sexually mature as males, spawn, then switch to a female phase. At age one year, these oysters enter a male phase followed again by a female phase.

TABLE 1.

Sex ratios and percent hermaphroditism in oyster species.

Family	Genus species	Number	Sex ratio (F:M)	% Hermaphrodite	Source
Ostreidae	*Crassostrea angulata*		1.00:0.79		Pelseneer 1926
	Crassostrea commercialis		0.05:1.00		Roughley 1933
			1.00:0.37		
	Crassostrea cucullata	333	1.00:1.00	0.9	Lasiak 1986
	Crassostrea gigas	120		0.8	Amemiya 1929
		776	1.00:0.81		Buroker 1983
		976	1.00:0.90		
		115	1.00:0.37		Mori 1982
		102	1.00:1.17		
		100	1.00:0.96		
		1377	1.00:1.00	0.23	Steele & Mulcahy 1999
	Crassostrea glomerata	121	2.00:1.00	0	Ansari & Ahmed 1972
	Crassostrea gryphoides	925	1.00:0.92		Durve 1965
	Crassostrea madrasensis		1.00:1.16		Stephen 1980
			1.00:1.11		
			1.50:1.00		
	Crassostrea paraibanensis		1.00:1.08		Singarajah 1980
			1.00:1.09		
	Crassostrea rhizophorae	226	1.00:2.32		Velez 1976
			1.00:3.00		Urpi et al. 1983
		1833	1.00:0.26		Littlewood & Gordon 1988
		536	1.00:1.00		Gruz et al. 1989
			1.00:1.00		Velez 1991
	Crassostrea tulipa		1.00:1.00		Yankson 1996
	Crassostrea virginica		1.00:1.00		Pelseneer 1926
		744	1.00:1.00		Burkenroad 1931
		1408	1.00:1.26	1.5	Menzel 1951
			1.00:1.00	<1	Kennedy 1982
			6.50:1.00		
			3.00:1.00		Heffernan et al. 1989
	Ostrea commercialis		2.70:1.00		Roughley 1933
	Ostrea equestris	469	1.00:7.43	2.6	Menzel 1955
		574	1.00:14.2	42.5	This Study
	Ostrea forkskali	43	2.60:1.00		Hulings 1986
	Ostrea iridescens	470	1.00:3.09	2	Fournier 1992
	Ostrea lurida	238		90.0	Coe 1932b
	Ostrea madrasensis		1.00:1.17		Stephen 1980
	Ostrea puelchana	109	1.00:0.79		Morricoui & Calvo 1989
		84	1.00:0.12		
	Saccostrea cucullata	497	1.78:1.00		Braley 1982
			1.00:1.38		Morton 1990
	Tiostrea chilensis	1542	1.00:0.15	96.1	Jeffs 1998
		1816	1.00:7.38	71.9	Jeffs & Hickman 2000

Most two-year-old oysters are females. In addition, female stages for this species have been observed developing within the gonads as the older gonadal material was releasing sperm (Cole 1942). Mann (1979) observed 29% hermaphroditic individuals in *O. edulis* while finding no hermaphrodites among *C. gigas*. For *O. lurida*, Coe (1932a) found that of 238 oysters 90% was hermaphrodites. Fournier (1992) also found a higher ratio of males to females (3:1) in *O. iridescens* with 2% hermaphroditism. Castro and Mattio (1987) described *O. puelchana* as a rhythmic consecutive hermaphroditic species. In *Tiostrea chilensis* populations, hermaphroditic individuals accounted for 72% and 96% of the oysters sampled in New Zealand (Table 1; Jeffs 1998, Jeffs & Hickman 2000, respectively).

The growth of crested oysters in Georgia was slow—a rate of 0.9 mm per month. The largest oyster observed was 36.1 mm in the September sample, which is half that of the maximum reported size (82 mm in shell length) of the crested oyster (Galtsoff & Merrill 1962). In the Gulf of Mexico region, Menzel (1955) showed that 12 oysters grew from a mean size of 13.1 mm to 29 mm in 9 months—a rate of 1.76 mm per month. The observed slow growth of the Georgia crested oyster is maybe a result of competition for both space and food. Crested oysters were gathered from a natural set in the Savannah River. When oysters were brought to the surface, relic *C. virginica* shells were already densely covered by crested oysters. No attempt to reduce the densities of the shells was made prior to or during the time that the crested oysters were held in pearl nets at the main dock on the Skidaway River. Thus, oysters that were collected in an overcrowded state remained in dense numbers throughout the sampling period. High stocking densities of bivalves result in slower growth rates (Sheldon 1968, Walker 1984, Holliday et al. 1993, Jensen 1993, Adams et al. 1994).

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OYSTER REEFS AS FISH HABITAT: OPPORTUNISTIC USE OF RESTORED REEFS BY TRANSIENT FISHES

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ABSTRACT Under the Magnuson-Stevenson Fisheries Management Act of 1996, current fisheries management practice is focused on the concept of Essential Fish Habitat (EFH). Application of the EFH concept to estuarine habitats relates directly to ongoing oyster reef restoration efforts. Oyster reef restoration typically creates complex habitat in regions where such habitat is limited or absent. While healthy oyster reefs provide structurally and ecologically complex habitat for many other species from all trophic levels including recreationally and commercially valuable transient finfishes, additional data is required to evaluate oyster reef habitats in the context of essential fish habitat. Patterns of transient fish species richness, abundance, and size-specific habitat use were examined along an estuarine habitat gradient from complex reef habitat through simple sand bottom in the Piankatank River, Virginia. There was no clear delineation of habitat use by transient fishes along this cline of estuarine habitat types (oyster reef to sand bar). Atlantic croaker (*Micropogonias undulatus*), Atlantic menhaden (*Brevoortia tyrannus*), bluefish (*Pomatomus saltatrix*), silver perch (*Bairdiella chrysoura*), spot (*Leiostomus xanthurus*), spotted seatrout (*Cynoscion regalis*), striped bass (*Morone saxatilis*), and weakfish (*Cynoscion nebulosus*) were found in all habitat types examined. In general, the smallest fish were found on the sand bar, the site with the least habitat heterogeneity. As habitat complexity increased along the gradient from oyster shell bar through oyster reef, transient fish size and abundance increased. Opportunistic habitat use by this suite of generalists relates variations in habitat quality as related to habitat-specific productivity and suggests that oyster reefs may be important but not essential habitat for these fishes.

KEY WORDS: habitat use, essential fish habitat, oyster reef, transient fish, Chesapeake Bay

INTRODUCTION

There is growing recognition by government and management agencies of the importance of habitat to maintenance and sustenance of marine fishery species. The Magnuson-Stevens Fishery Conservation and Management Act of 1996 (Public Law 94-265) as amended by the Sustainable Fisheries Act established the concept of Essential Fish Habitat and provided for the management and protection of such habitat under the auspices of the National Marine Fisheries Service (Benaka 1999). Essential Fish Habitat (EFH) was defined as "those waters and substrate necessary for fish for spawning, feeding or growth to maturity". Under the law, "finfish, molluscs, crustaceans, and all other forms of marine animal and plant life other than marine mammals and birds" are protected. While protection of marine habitats is certainly needed, the scale of the Magnuson-Stevens Act, as established by its terminology, renders application of the law on a practical level next to impossible. The Magnuson-Stevens Act provides a means to classify fish habitats as essential (absolutely necessary per Webster's Dictionary 1983) but offers no opportunities to distinguish gradations in fish habitat quality. Functionally, the only habitat absolutely necessary for fish is reasonably clean water.

As restoration efforts in Chesapeake Bay and other estuaries continue to focus on oyster reef reconstruction and rehabilitation, the nature and importance of oyster reefs as habitat (the place where an animal lives sensu Odum 1971) bears further investigation. Oyster reefs, three dimensional structures created and maintained by living oysters (*Crassostrea virginica*), were historically a principal habitat type in shallow portions of estuaries such as Chesapeake Bay. The chronic decline of oyster populations in the 20th century due to a combination of overfishing, disease, and habitat degradation has reduced oyster populations and virtually eliminated natural oyster reef structures in Chesapeake Bay (Hargis 1999). Oyster reefs are ecologically valuable as habitat for oysters as well as a diverse suite of resident benthic fauna (e.g., oysters, barnacles, mussels, polychaetes, crabs, naked gobies (*Gobiosoma bosc*); Wells 1961, Bahr & Lanier 1981, Meyer & Townsend 2000) and recreationally and commercially valuable transient fishes (e.g., striped bass (*Morone saxatilis*), bluefish (Pomatomus saltatrix), Atlantic croaker (*Micropogonias undulatus*), spot (*Leiostomus xanthurus*); Breitburg 1999, Coen et al. 1999, Harding & Mann 1999, Posey et al. 1999).

The ecological function of oyster reef habitats is dependent upon both structural and ecological features inherent in living reef communities, namely the oyster's benthic-pelagic coupling capabilities and the resulting production of hard shell substrate (Coen et al. 1999, Mann 2000, Coen & Luckenbach 2000). Restored oyster reef communities should follow an ecological progression towards climax or stability in numbers and species (Sale 1980) over time. Various measures of reef community development have been proposed including abundance of adult oysters in relation to local (within 1 km) natural (not restored) oyster populations (Harding & Mann 1999) and larval production in relation to adult abundances for primary and secondary trophic levels of reef residents (Harding & Mann 2000).

There is merit in examining the use of restored oyster reef habitat by transient finfish particularly in relation to local non-reef habitats. Burchmore et al. (1985), Breitburg (1999), and Harding and Mann (1999) describe transient reef fishes as mobile schooling species that are found over a wide range of habitats including reefs. Descriptions of fish species richness in relation to oyster reefs have been made by Wenner et al. (1996), Nestlerode et al. (1998), Coen et al. (1999), Harding and Mann (1999), Minello (1999) and Posey et al. (1999) with the continuing observation that oyster reefs are home to diverse assemblages of transient fishes.

National Marine Fisheries Service guidelines (62 FR 66531, 1997) suggest delineation of EFH in light of four hierarchical information levels (Minello 1999): presence/absence data (Level 1), distribution and abundance (density) information (Level 2), functional relationships between species and habitats: reproduc-

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tion, growth, and survival (Level 3), and habitat-specific fish production (Level 4). Current designations of habitat as EFH rely on basic information as provided by Level 1 and 2 in the absence of comprehensive data sets addressing information Levels 3 and 4 (Able 1999, Minello 1999). The objectives of this paper are to compare the transient finfish assemblages associated with a gradient of habitats ranging from hard sand bottom to oyster reef within the same estuary and relate the observed patterns of species richness (Level 1), abundance (Level 2), and size-specific habitat use (Level 3) to habitat classifications sensu EFH.

Study Site

Field work was conducted in the Piankatank River, Virginia at three sites (Fig. 1): Palace Bar oyster reef, an oyster shell bar (Ginney Point), and a sand bar (Roane Point). Palace Bar reef is an intertidal oyster reef (210 × 30 m, reef depth range of 0.5 m above mean low water (MLW) to 3 m below MLW) adjacent to the historic Palace Bar oyster grounds. Palace Bar reef was built in 1993 by the Virginia Marine Resources Commission (VMRC) Shellfish Replenishment program as a series of 18 shell mounds centered on and around an east-west centerline 300 m long (Mann et al. 1996). Approximately 70% of the reef (0.63 ha) is composed of oyster shell, while the remaining area (0.27 ha) is crushed clam shell. Palace Bar reef has supported oyster densities similar to those observed on natural (i.e., not constructed) oyster bars in the Piankatank River since 1997 (Harding & Mann 1999, R. Mann, unpublished data). The Ginney Point site is a flat oyster shell bar with a depth range of 2.5–3 m below MLW (Fig. 1). The Roane Point site includes a sand bar (depth range 1.5–2 m below MLW) south and inshore of Palace Bar reef (Fig. 1). Mean tidal range in the Piankatank River is approximately 0.4 m and maximum tidal current at these sites is approximately 0.12 m^{-s} (Chen et al. 1977).

MATERIALS AND METHODS

Transient fishes were sampled using multi-panel experimental gill nets (one 30.5 m × 1.8 m and two 30.5 m × 3.0 m nets all with one 7.6 m panel each of stretch square mesh monofilament of 57.2, 63.5, 73.0, and 76.2 mm) deployed such that the entire water column was sampled (e.g., the smallest net at Roane Point, the shallowest site). Nets were deployed in a straight line parallel to tidal flow at each site. All fishes were removed from the gill nets identified, sacrificed, and measured (total length to the nearest mm) resulting in species-specific presence/absence, abundance, and size estimates across a gradient of habitat types (oyster reef to sand bar).

Transient fishes were collected during 8 thirty-six hour sampling events completed from May through September on the new and full moon (May 22–23, June 5–6, June 19–20, July 2–3, July 17–18, August 4–5, August 18–19, and September 2–3, 1997). Sampling periodicity incorporated complete diurnal and tidal cycles as well as seasonal progression. During each sampling sequence, reef and non-reef sites were sampled at three-hour intervals corresponding to changes in tidal stage for thirty-six consecutive hours. Water temperature and salinity were recorded weekly from May through September 1997 at Ginney Point and Palace Bar reef (Fig. 2).

Data Analyses

Significance levels for all statistical tests were established at $p = 0.05$ *a priori*. Bartlett's test for homogeneity of variance and the Ryan-Joiner test for normality were used prior to parametric analyses. When appropriate, Tukey's tests were used for post-hoc multiple comparisons.

Piankatank River Temperature and Salinity Data

Water temperature and salinity data for Ginney Point and Palace Bar reef were transformed (natural logarithm) to meet the assumptions of homogeneity of variance and normality prior to analyses with ANOVA.

Species-specific Abundance Data

Only the six species that were numerically dominant ($n > 5$ individuals per station for each of the three sites) were used in these analyses. For each species, the number of fish caught per gill net deployment were compared with an ANOVA using site, day of the year, and time of day as factors. Data for bluefish, striped bass, and weakfish met both the assumptions of homogeneity of variance and normality after transformation with the reciprocal transformation (Zar 1996). Data for croaker and spot satisfied the assumptions of homogeneity of variance and normality after logarithmic transformation. Counts for Atlantic menhaden satisfied the assumption of homogeneity of variance with the reciprocal transformation but not normality regardless of the transformation (log + 1, ln + 1, sqrt + 1, reciprocal).

Fish Assemblage-Habitat Relationships

Transient fish species abundance associations were compared across sites using detrended correspondence analysis (DCA). DCA was used as a descriptive tool to characterize the fish assemblages observed at each site on the basis of abundance. DCA ordinations spatially aggregate similar samples and separate dissimilar ones on the basis of species abundances within a sample. All DCA analyses (CANOCO for Windows version 4.0 1998) were detrended with second order polynomials (per ter Braak 1995) to avoid potential loss of gradient information during the detrending procedure (Minchin 1987). Species-samples biplots were made using CANODRAW software (version 3.1, Similauer 1998).

Species-specific Length Data

Total lengths (mm) for the six numerically dominant species were compared with species-specific one-way ANOVAs using site as a factor.

RESULTS

Analyses

Piankatank River Temperature and Salinity Data

Neither water temperatures nor salinity values were significantly different among sampling sites in 1997 (ANOVA, $p < 0.05$). Water temperature and salinity conditions observed in the Piankatank River during 1997 were similar to those observed during 1993–96 (Fig. 2, R. Mann, unpublished data).

Species-specific Abundance Data

Fourteen different transient fish species were observed in gill net collections from Palace Bar reef (Table 1). Ten of these fourteen species were observed at Ginney Point (oyster shell bar) and nine were observed at Roane Point (sand bar). Atlantic croaker, Atlantic menhaden, bluefish, spot, striped bass, and weakfish were the most abundant fish species at all three sites (Table 1).

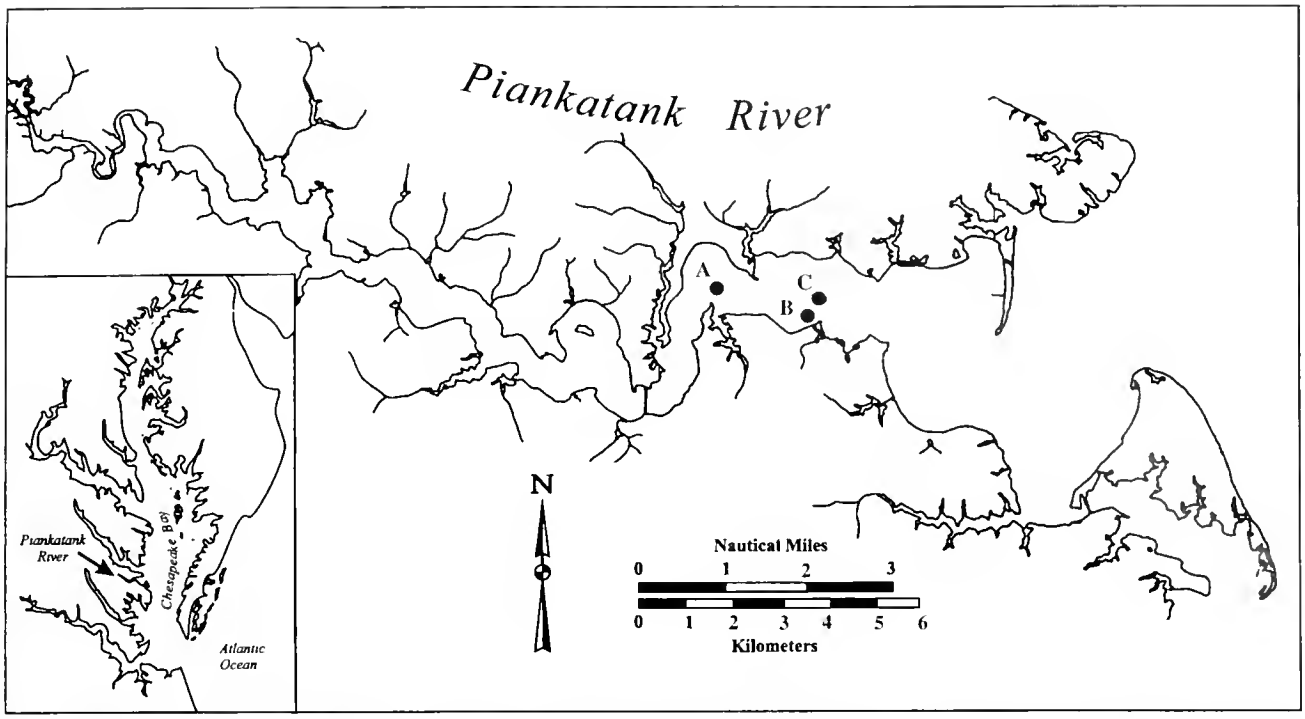

Figure 1. Map of the Piankatank River in relation to the Chesapeake Bay showing sampling locations after Harding and Mann (1999). Palace Bar reef (C), Ginney Point (an oyster shell bar, A) and Roane Point (a sand bar, B) were sampled to provide data for reef vs. non-reef habitat comparisons.

Abundances of Atlantic croaker, Atlantic menhaden, and striped bass were significantly greater at sites with oyster shell substrate (Palace Bar reef and Ginney Point) than at the sand bar site (Roane Point) but there was no significant difference in abundance of these three species between the oyster reef and the oyster bar (Table 2; ANOVA, Tukey test, $p < 0.05$; Figs. 3, 4, and 7). Bluefish were significantly more abundant at the oyster reef than at any other site (Table 2; ANOVA, Tukey test, $p < 0.05$). Spot were significantly more abundant at the oyster bar than at either the oyster reef or the sand bar (Table 2; ANOVA, Tukey test, $p < 0.05$). Weakfish abundance was low relative to the other species and similar across all three sites (Table 2; ANOVA, Tukey's test, $p < 0.05$).

In general, fish abundance increased at night across all sites. Atlantic croaker, bluefish, and spot were significantly more abundant from dusk to dawn (2000–0800) than during the day (Figs. 3, 5, 6; ANOVA, Tukey's test, $p < 0.05$). Striped bass were significantly more abundant from dusk to dawn than at mid-day (1200–1600; Fig. 7, ANOVA, Tukey test, $p < 0.05$). Atlantic menhaden and weakfish were significantly more abundant during darkness (2000–0800); abundances observed between midnight and 0400 were higher than at any other time for both menhaden and weakfish (Figs. 4 and 8; ANOVA, Tukey's test, $p < 0.05$).

Fish abundances varied seasonally. Bluefish were significantly more abundant in May and September than from June to August (Fig. 5, ANOVA, Tukey's test, $p < 0.05$). Striped bass and Atlantic menhaden were significantly more abundant in May than at any other time during the year and more abundant in late June than during late July and August (Figs. 4 and 7; ANOVA, Tukey's test, $p < 0.05$). Weakfish were significantly more abundant in late July (Fig. 8, ANOVA, Tukey's test, $p < 0.05$). Atlantic croaker abundance was significantly greater during July and early August while

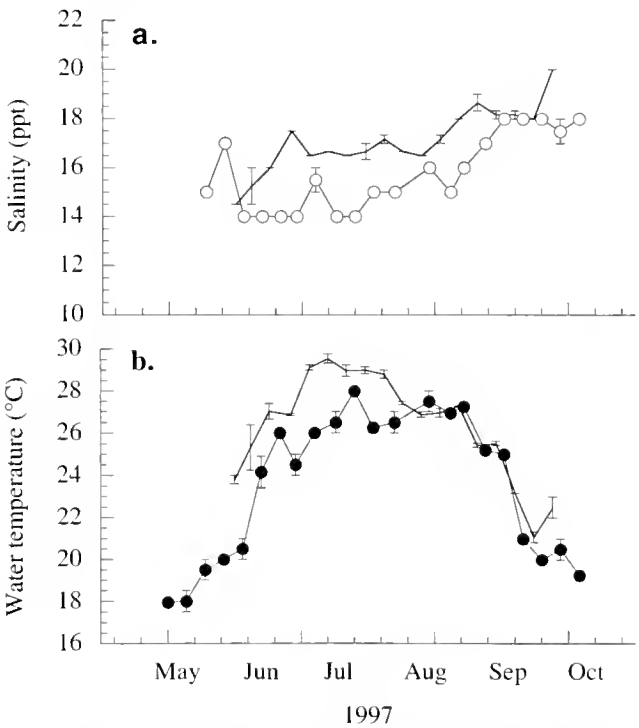

Figure 2. a-Mean salinity (ppt) and b-water temperature (°C) values (±standard error) for Ginney Point and Palace Bar reef, Piankatank River, Virginia from May through September 1997 after Harding and Mann (2001). Data from these two sites were averaged since there was no significant difference in temperature or salinity between sites (ANOVA, $p < 0.05$). Reference mean values for temperature and salinity data from 1993–1996 are plotted with a solid line (± standard error). 1997 data are indicated by lines with symbols (± standard error).

TABLE 1.

Total number of transient fish species collected with gill nets at Palace Bar oyster reef, Ginney Point (oyster bar), and Roane Point (sand bar), Piankatank River, Virginia during 8 thirty-six-hour stations conducted from May 22 to September 3, 1997.

Common Name	Scientific Name	Palace Bar Reef	Ginney Point	Roane Point
Atlantic croaker	*Micropogonias undulatus*	121	120	70
Atlantic menhaden	*Brevoortia tyrannus*	480	455	195
Bluefish	*Pomatomus saltatrix*	65	35	20
Spot	*Leiostomus xanthurus*	221	258	150
Striped bass	*Morone saxatilis*	62	98	10
Weakfish	*Cynoscion regalis*	14	11	7
Blueback herring	*Alosa aestivalis*	3	5	4
Butterfish	*Peprilus triacanthus*	1	0	0
Cownose ray	*Rhinoptera bonasus*	1	0	0
Gizzard shad	*Dorosoma cepedianum*	1	0	0
Hog choker	*Trinectes maculatus*	3	0	0
Silver perch	*Bairdiella chrysoura*	38	3	2
Spotted seatrout	*Cynoscion nebulosus*	4	8	4
Summer flounder	*Paralichthyes dentatus*	0	2	0

spot were significantly less abundant in August (Figs. 3 and 6; ANOVA, Tukey's test, $p < 0.05$).

Fish Assemblage-Habitat Relationships

A detrended correspondence analysis (DCA) using all samples and all species (Fig. 9) aggregated all but one of fourteen species (summer flounder) and all but two of 231 samples (the two samples containing flounder) from all three sites along a single axis virtually on top of each other. This cohesive spatial grouping indicates strong similarity of most species and samples across all sites. Axis I describes a gradient in diurnal light levels moving from left (dark) to right (light). Axis II represents a seasonal gradient in water temperatures moving from bottom (lower water temperatures) to top (warmest water temperatures). The variance explained by the axes was 21.6% (Axis I) and 38.2% (Axis II).

If rare species or species where the total number of fish observed across all three sites was less than fifteen are removed from the analysis, eight species remain (Table 1). A second DCA using only these eight species in the gill net samples (Fig. 10) shows a lack of spatial aggregation of samples by site in ordination space as would be expected by site-specific fish assemblages. Thus, the samples from all three sites show a ubiquitous distribution. Axis I represents a gradient in diurnal light levels moving from left (dark) to right (light). Axis II represents a seasonal gradient in water temperatures moving from bottom (lower water temperatures) to top (warmest water temperatures). Fishes that were more abundant from dusk to dawn during late May, June, and early September (spot, bluefish) are grouped toward the middle of the plot to the left of fishes that were more abundant from dusk to dawn in July (silver perch, weakfish; Fig. 10). Primarily nocturnal species (Atlantic menhaden and spotted seatrout) are grouped to the left (dark) side of Axis I. Striped bass were most abundant in May and early June during daylight hours as indicated by their position in the lower right corner of the plot (Fig. 10). Atlantic croaker were frequently caught between dawn and dusk during the warmer months as indicated by their position in the upper right corner of the plot (Fig. 10). The variance explained by the axes was 28.5% (Axis I) and 48.8% (Axis II).

Species-specific Length Data

Atlantic croaker, Atlantic menhaden, and striped bass observed at Palace Bar reef are significantly larger than fishes of these species observed from either the oyster bar or the sand bar (Table 3; ANOVA, Tukey's test, $p < 0.05$). Spot from the oyster bar are larger than spot from any other site (Table 3; ANOVA, Tukey's test, $p < 0.05$). Bluefish from the reef are slightly but not significantly larger than fish from other sites and weakfish from all sites are of similar length (Table 3; ANOVA, Tukey's test, $p > 0.05$).

DISCUSSION

There was no clear delineation of habitat use by transient fishes along a gradient of estuarine habitat types (oyster reef to sand bar). Atlantic croaker, Atlantic menhaden, bluefish, silver perch, spot, spotted seatrout, striped bass, and weakfish were found in all habitat types examined. The ubiquitous distribution of these common species indicates a lack of site-specific fish assemblages in these habitats. It is unreasonable to expect site-specific groupings of

TABLE 2.

Summary of ANOVA results (p-values) for species-specific abundance (number of a species collected per gill net deployment) of the six most abundant transient fish species observed in the Piankatank River in relation to site, day of the year, and time of day. Asterisks indicate results that were significant at the $p < 0.05$ level.

Factor	df	Atlantic Croaker	Atlantic Menhaden	Bluefish	Spot	Striped Bass	Weakfish
Site	2	0.01*	0.01*	0.02*	<0.01*	<0.01*	0.12
Day of the year	7	0.01*	<0.01*	0.02*	<0.01*	<0.01*	<0.01*
Time of day	5	<0.01*	<0.01*	<0.01*	<0.01*	0.01*	<0.01*

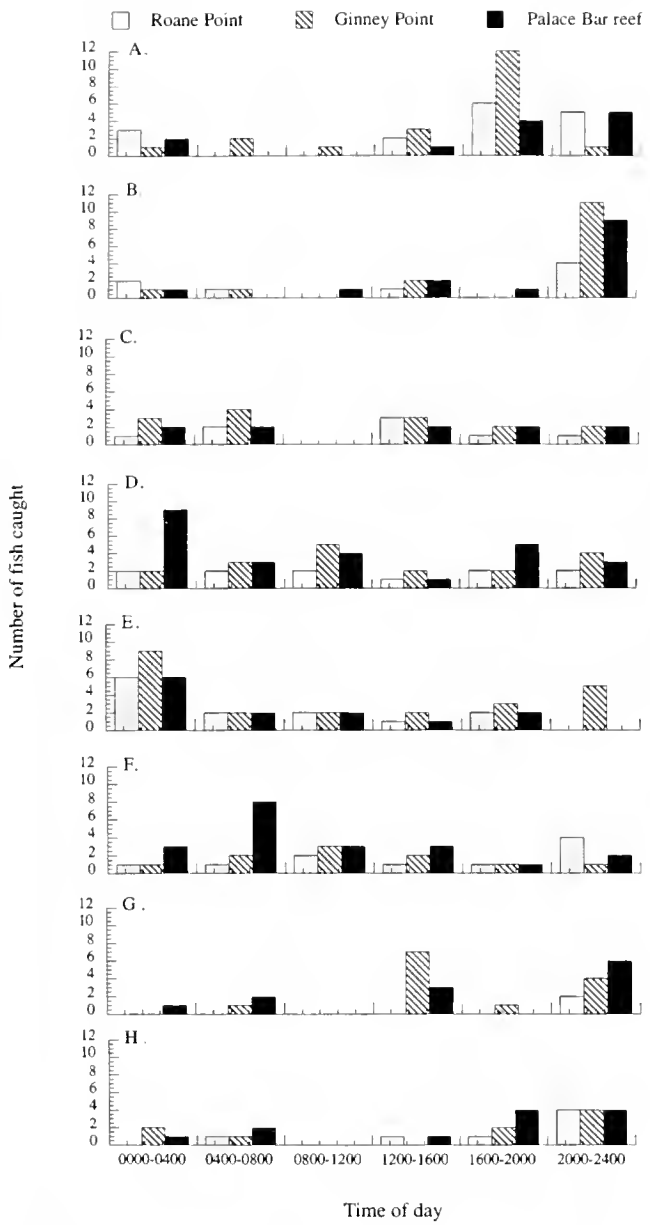

Figure 3. Species-specific abundance for Atlantic croaker in relation to time of day and day of the year for A.) May 22–23, B.) June 5–6, C.) June 19–20, D.) July 2–3, E.) July 17–18, F.) August 4–5, G.) August 18–19, and H.) September 2–3, 1997.

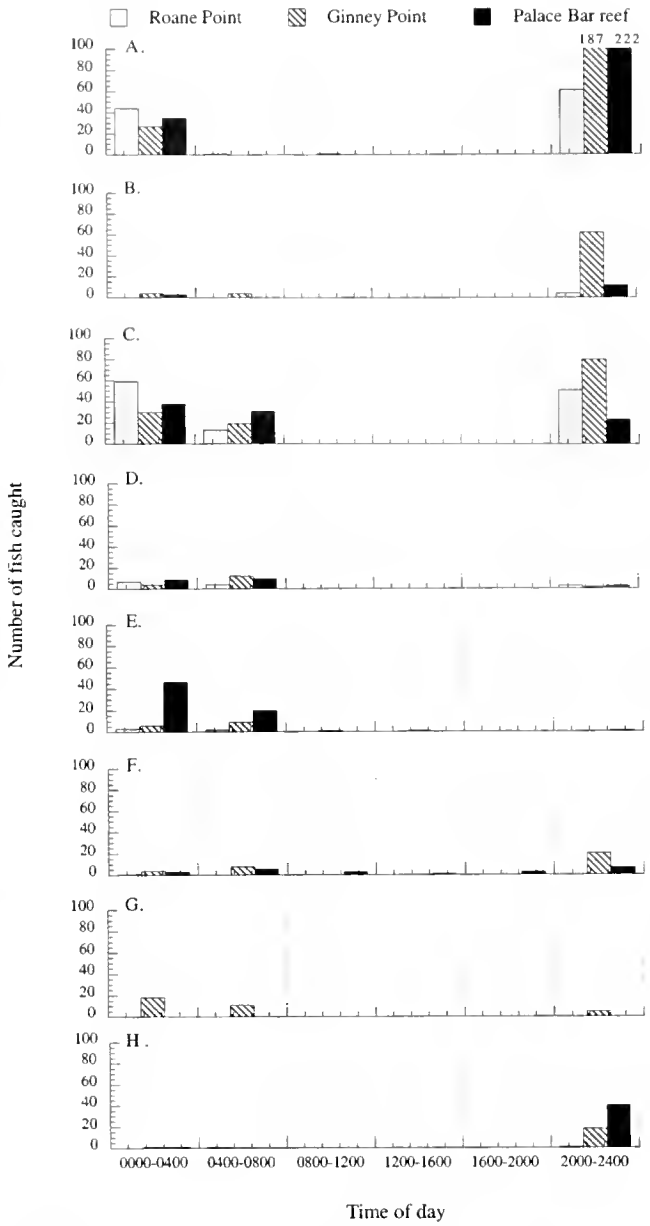

Figure 4. Species-specific abundance for Atlantic menhaden in relation to time of day and day of the year for A.) May 22–23, B.) June 5–6, C.) June 19–20, D.) July 2–3, E.) July 17–18, F.) August 4–5, G.) August 18–19, and H.) September 2–3, 1997.

generalist species such as these that are opportunistically using available habitat. It is more likely that habitat use by these eight fish species relates to variations in habitat quality indicated by habitat-specific productivity.

In general, the smallest fish are found on the sand bar, the site with the least habitat heterogeneity. As habitat complexity increases along the gradient from oyster shell bar through oyster reef, transient fish size and abundance increases. The oyster reef may have relatively higher food availability, a wider diversity of food types because of increased habitat heterogeneity, or greater abundance of high quality food relative to other habitat types. Dietary analyses on bluefish (Harding & Mann 2000) and striped bass (Harding & Mann, unpublished data) from these sites corroborate these functional relationships between reef habitats and transient fishes. Bluefish from sites with oyster shell substrate consume more teleosts than bluefish from the sand bar (Harding & Mann 2001). Bluefish from Palace Bar reef consume a wider diversity of prey items than fish from other sites (Harding & Mann 2001) while reef striped bass consumed more teleosts in general and naked gobies in particular than fish from other sites (Harding & Mann, unpublished data). In other words, the observed differences in fish abundance and size across habitat types may relate to habitat productivity as enhanced by ecological and structural complexity.

Presence/absence and abundance data from this study demonstrate that these transient finfish employ generalist lifestyle strategies (Sale 1980) and are opportunistically using the range of available habitat on a local scale. The habitats of interest herein

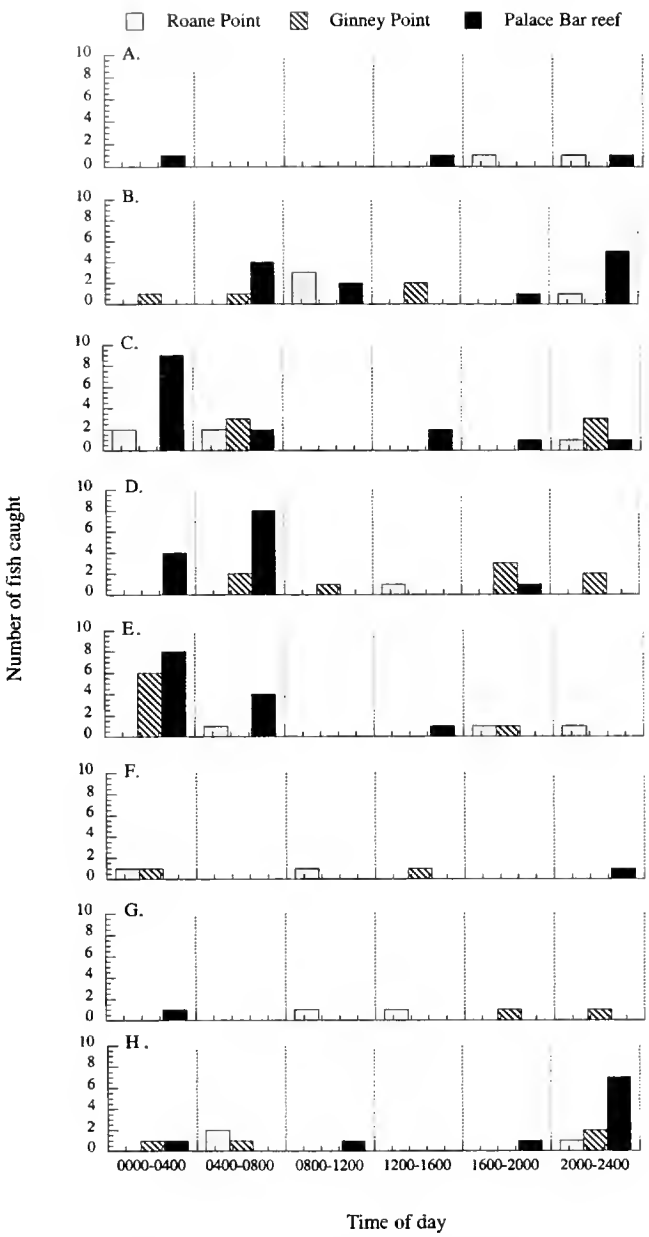

Figure 5. Species-specific abundance for bluefish in relation to time of day and day of the year for A.) May 22–23, B.) June 5–6, C.) June 19–20, D.) July 2–3, E.) July 17–18, F.) August 4–5, G.) August 18–19, and H.) September 2–3, 1997.

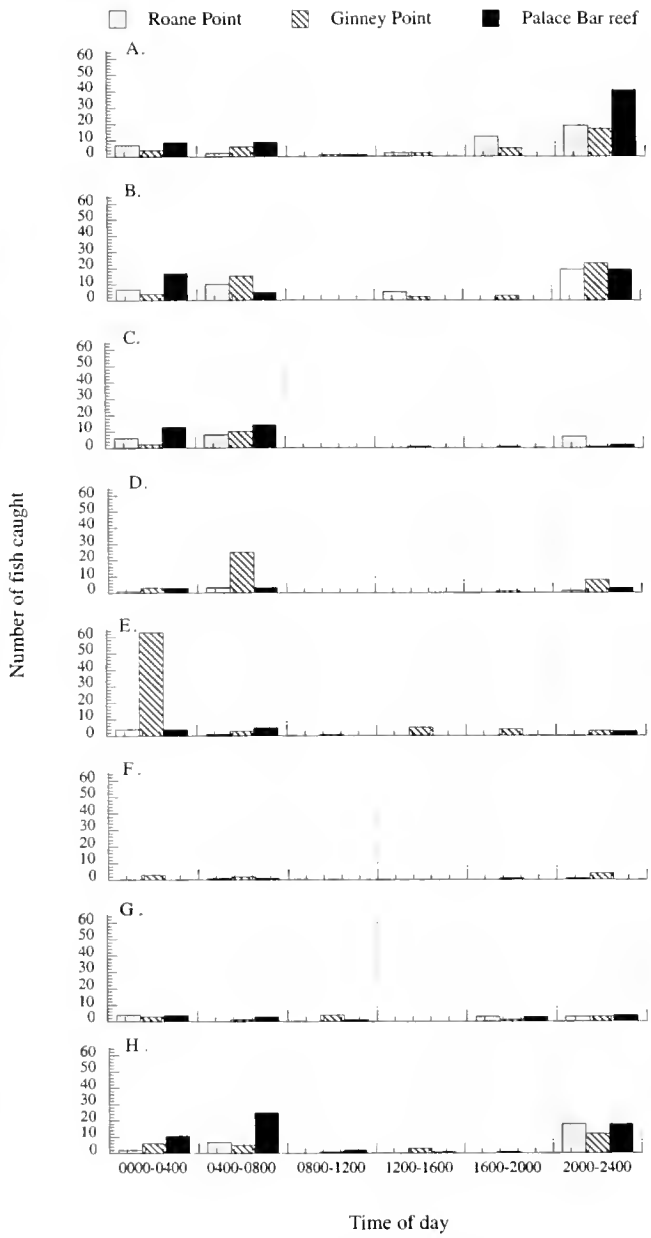

Figure 6. Species-specific abundance for spot in relation to time of day and day of the year for A.) May 22–23, B.) June 5–6, C.) June 19–20, D.) July 2–3, E.) July 17–18, F.) August 4–5, G.) August 18–19, and H.) September 2–3, 1997.

represent a gradient or cline of habitat complexity commonly observed in temperate estuaries; namely a cline moving from simple, unstructured hard sand bottom habitats through hard bottom shell habitats with little vertical relief culminating in complex, three-dimensional reef structures created and maintained by oysters. These biogenic reef structures naturally ranged in size from acres to hectares and historically were dominant habitat types in Chesapeake Bay.

This gradient of habitat types is a temperate analog to tropical coral reef systems ranging in scale from patch reefs through much larger reef systems (e.g., the Great Barrier Reef). The transient fish communities associated with temperate and tropical reef habitats are composed primarily of generalists that will opportunistically use available habitat (Sale 1980, Ebling & Hixon 1993, Roberts 1993). The structural and ecological complexity of reef habitats makes them attractive foraging habitat for transient finfish as well as aggregation sites. Historically, shallow portions of Chesapeake Bay were characterized by a mosaic of habitat types including biogenic structure ranging from seagrass beds to oyster reefs extending across spatial scales ranging from kilometers to 10s of kilometers. The development of large biogenic reef structures was facilitated by the evolution of the Chesapeake Bay estuary (Hargis 1999). The parallel development of the Bay's fish fauna favored transient fishes with broad habitat and dietary requirements (generalists) that were able to opportunistically use the dynamic estuarine habitat. These fishes successfully use the modern Chesapeake habitat in spite of relatively recent habitat alterations, namely the decline of both seagrass beds and oyster reefs during the late 20th century.

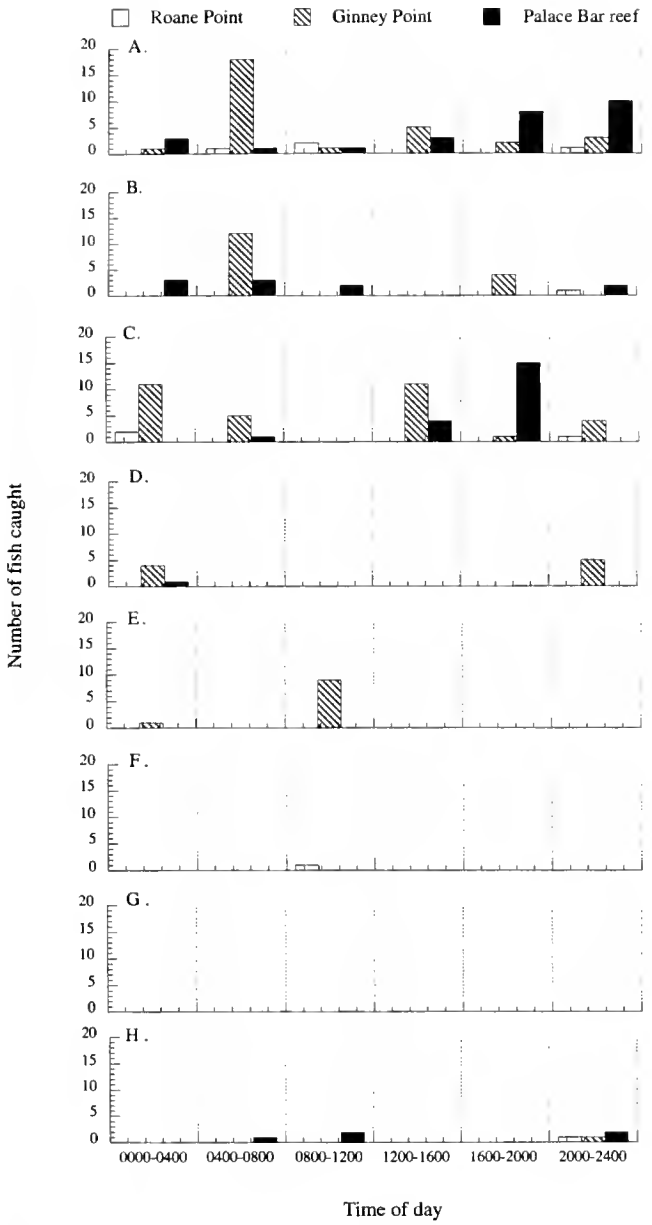

Figure 7. Species-specific abundance for striped bass in relation to time of day and day of the year for A.) May 22–23, B.) June 5–6, C.) June 19–20, D.) July 2–3, E.) July 17–18, F.) August 4–5, G.) August 18–19, and H.) September 2–3, 1997.

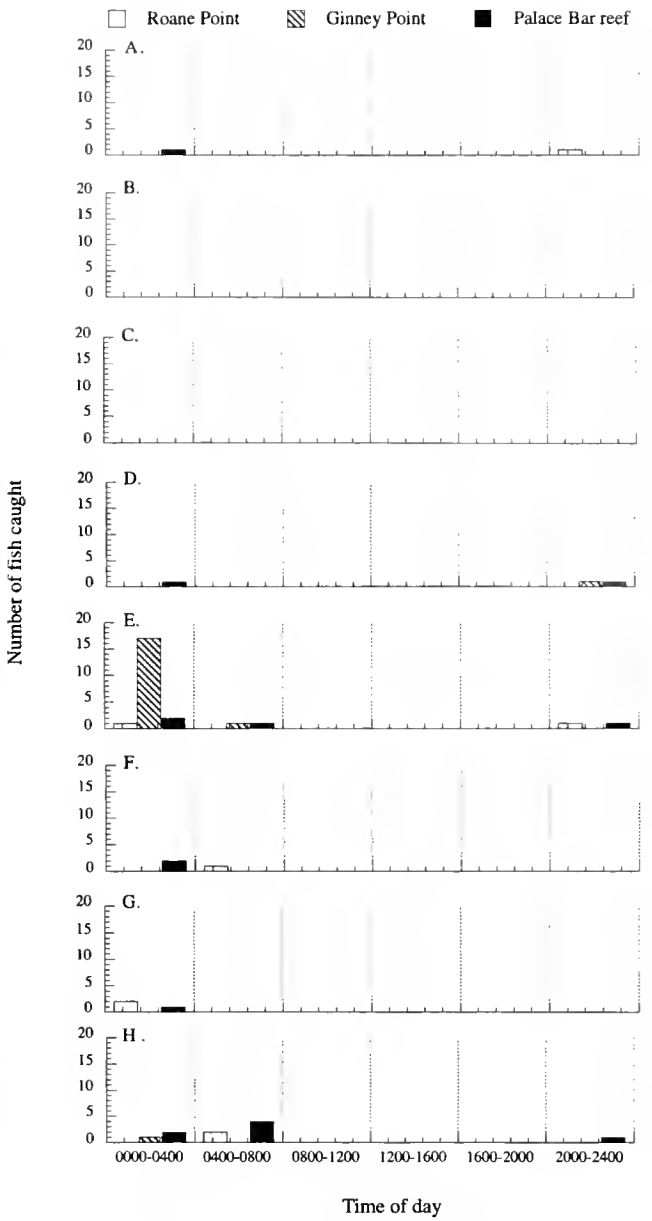

Figure 8. Species-specific abundance for weakfish in relation to time of day and day of the year for A.) May 22–23, B.) June 5–6, C.) June 19–20, D.) July 2–3, E.) July 17–18, F.) August 4–5, G.) August 18–19, and H.) September 2–3, 1997.

Previous discussions of oyster reef habitats as essential fish habitat for transient finfish (Breitburg & Miller 1998, Coen et al. 1999) have examined fish species richness data from a geographic range of oyster reef habitats including both natural and restored reefs of varying ages. Coen et al. (1999) suggest that the use of oyster reef habitats by transient fish species "portends the reef habitats' importance as essential fish habitat, but many functional relationships remain to be evaluated". This study presents a unique comparison of transient fish use of oyster reefs in relation to other locally available habitat types and is the first to provide data to describe fish habitat use at Level 1 (presence/absence), Level 2 (abundance) and Level 3 (size) levels of EFH designation. These data clearly show that these transient generalist fishes do not rely exclusively on oyster reef habitats. From a local historical perspective, the continued presence of these species in the lower Chesapeake in the absence of natural oyster reefs for the past 20+ years (Hargis 1999) is an obvious indicator that oyster reef habitat is not essential for these opportunistic fishes.

The habitat value of oyster reefs to transient fishes is much more complicated than a binary distinction (essential or not essential). Evaluations of oyster reefs as fish habitat must consider reefs in the context of locally available habitat types (per Minello 1999; this study) if accurate descriptions of habitat importance are to be made, particularly for transient finfish species. Continued examination of the functional ecological relationships between oyster reefs and the trophic communities that they support will provide data on which to base habitat distinctions at all four levels of EFH description and related resource management decisions. Gradients

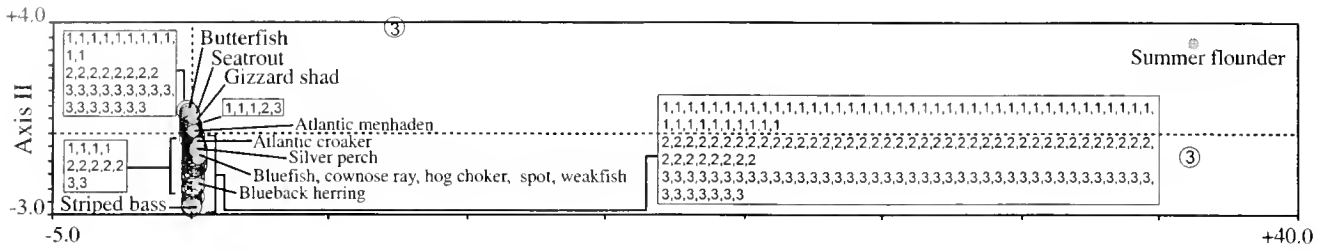

Figure 9. Species-sample biplot for detrended correspondence analyses (DCA) describing transient finfish assemblages and species abundances across a gradient of habitat types ranging from sand bar through three dimensional oyster reef. Fourteen species from two hundred and thirty-one samples collected at Palace Bar reef (1), Roane Point (2), and Ginney Point (3) with gill nets are presented. Axis I represents a gradient in diurnal light levels moving from left (dark) to right (light). Axis II represents a seasonal gradient in water temperatures moving from bottom (lower water temperatures) to top (warmest water temperatures).

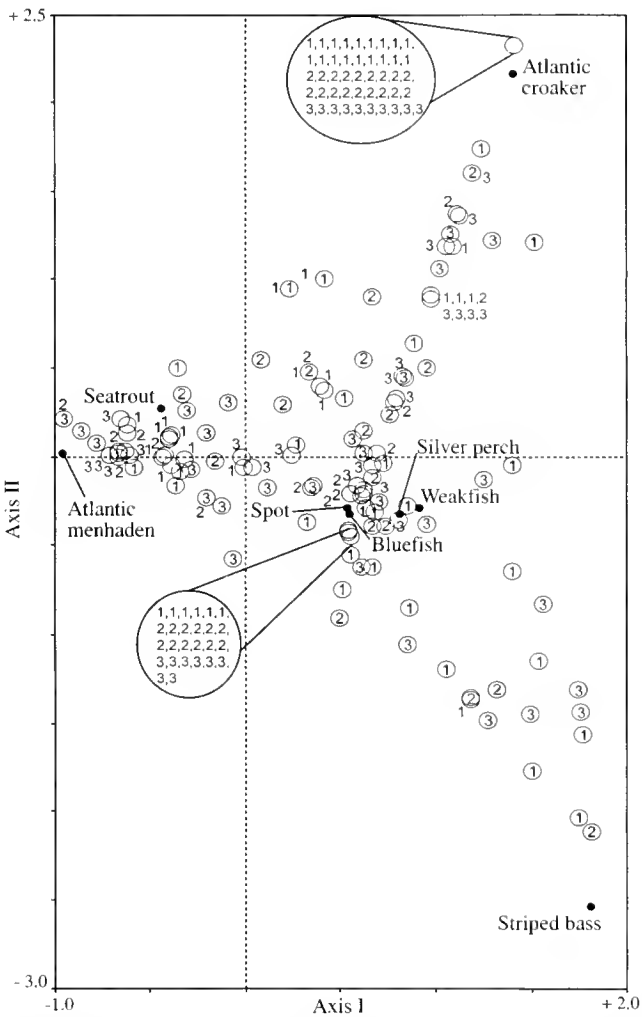

Figure 10. Species-sample biplot for detrended correspondence analyses (DCA) of common fish species across a gradient of habitat types ranging from sand bar through three dimensional oyster reef. Eight species from 201 samples collected at Palace Bar reef (1), Roane Point (2), and Ginney Point (3) with gill nets are presented. Axis I represents a gradient in diurnal light levels moving from left (dark) to right (light). Axis II represents a seasonal gradient in water temperatures moving from bottom (lower water temperatures) to top (warmest water temperatures).

TABLE 3.

Average total length (mm) of the most common transient fish species (standard error) collected with gill nets at Palace Bar oyster reef, Ginney Point, and Roane Point, Piankatank River, Virginia. Site-specific species total lengths were compared with species-specific ANOVAs. Horizontal lines under site-specific species average lengths values indicate sites where statistically similar sizes of a particular species were observed (ANOVA, Fisher's test; $p < 0.05$).

Fish	Palace Bar Reef	Ginney Point	Roane Point
Atlantic croaker	311.6 (3.3)	295.1 (2.8)	290.2 (3.9)
Atlantic menhaden	262.1 (1.9)	246.8 (1.6)	239.7 (1.0)
Bluefish	307.1 (5.7)	298.3 (5.8)	297.1 (6.1)
Spot	199.1 (1.6)	205.1 (1.4)	198.5 (1.7)
Striped bass	294.5 (5.1)	261.7 (3.3)	278.6 (10.7)
Weakfish	286.4 (8.9)	312.4 (20.8)	302 (20.7)

in physical habitat complexity relate to gradients in habitat productivity and thus habitat value or importance. A gradient of terms to describe habitat value that reflects the ecological value of a habitat would be a more realistic tool for habitat distinction. Given their physical and trophic complexity, oyster reefs are important habitat for transient estuarine finfish, however, on the basis of these data, we question the use of term "essential" with regard to oyster reef habitats given the generalist nature of the transient fish species that use these habitats. We suggest that oyster reef habitats are not essential for these fishes but that oyster reef habitats are of higher quality than other locally available estuarine habitat types and thus are better or perhaps even optimal for these fish in terms of growth, reproductive success, and survival.

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IMPACT OF REPEATED DREDGING ON A DELAWARE BAY OYSTER REEF

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ABSTRACT The impact of commercial dredging on an oyster reef was evaluated at four sites chosen on New Beds, one of the most important commercial oyster beds in Delaware Bay. Dredging occurred on two of these sites in late October 1999, early and late November 1999, April 2000, and July 2000. Dredging was conducted according to standard industry procedures. Each day, dredging was continuous during approximately an 8-h period. Both one-dredge and two-dredge boats were used. Market-size oysters were culled and sacked in the standard manner. Total dredge coverage for the study was about 240,000 m^2 on each experimental site. The most heavily dredged areas were completely covered by the dredge 4 to 6 times during the study. Two 8-h dredging events within a 10-day period produced barely detectable changes in the oyster population. Minor chipping and abrasion of the shell increased in frequency, but no other discernible impacts were found. Over the 10-mo study that included five dredging events, many of the taphonomic indicators of dredge damage showed time-dependent trends that differed between control and experimental sites. However, these effects were limited mostly to minor chipping and indications of abrasive wear, rather than the more serious aspects of shell damage defined as major chipping, breakage, cracking, and shell perforation. A variety of population health indicators were assayed during the study, including the ratio of live oysters to boxes, condition index, *Perkinsus marinus* infection intensity, and oyster size-frequency distribution. These indicators should have monitored growth, disease pressure, and mortality. Essentially no significant effects could be discerned for any of these measures. Over a very long time, dredging may significantly influence oyster bed physiography and community structure. However, once the bed has become a fished bed, this study suggests that moderate dredging that results in a yearly swept area of no more than four times the area of the bed is unlikely to result in significant further impact on the oyster populations living there.

KEY WORDS: oyster, fishery, dredging, fishing impact, population structure, tophonony

INTRODUCTION

Fishing with bottom-tending gear such as dredges and otter trawls may impact the benthic community in a number of ways. Normally, the impacts are negative to some degree (Collie et al. 1997, Jennings & Kaiser 1998, Thrush et al. 1998). For sessile shellfish in which the efficiency of the dredge is low (Andrews 1988, Giguère & Brulotte 1994, Vølstad et al. 2000), the negative impacts may extend to the target species (Hall 1994, Lenihan & Petersen 1998, Robinson & Richardson 1998). Decreases in growth rate, increases in shell damage, and increased mortality are among the possible impacts that might occur to target shellfish species. In rarer cases, positive impacts may occur. For the oyster, for example, dredging may enhance spat settlement by turning over the shell, and the increased efficiency of capture often observed with repeated dredging should reduce the overall impact of the fishery on routinely fished beds.

Development of a direct-market fishery in the last half of the 1990s on what were historically the Delaware Bay seed beds occurred as a response to the development of Dermo disease, caused by the protozoan *Perkinsus marinus* (Powell et al. 1997). The development of Dermo disease reduced survival on downbay leased grounds so that the historical fishery, based on transplant of seed from upbay seed beds downbay to leased grounds, was no longer profitable. The direct-market fishery, essentially a managed wild fishery, has substantially increased the total amount of dredging on the seed beds that originally supplied seed to the downbay leases.

Yearly stock assessments conducted since the direct-market fishery was initiated have documented a more or less steady decline in oyster abundance on the most heavily fished beds (HSRL 2000). The extent to which this decline is due to the impact of repeated dredging rather than the simple removal of oysters for market is unknown. The general question of the impact of dredging on oyster reefs has received considerable attention over the years. Some have concluded that the impact has been substantial (Marshall 1954, Haven & Whitcomb 1983, McCormick-Ray 1998); others have concluded that the impact has been inconsequential or even positive (Powell et al. 1995). These studies evaluated long-term changes in reef geography, for the most part. In no case has a study been conducted to directly assess the damage to the oyster population caused by repeated dredging of a reef as a normal course of fishing.

Unfished oyster reefs are typified by a complex three-dimensional structure of oyster clumps (Powell et al. 1987, Wilson et al. 1988). Fishing, regardless of the gear used, results in a substantial change in reef micro-topography from this pristine state. However, once the lower-microrelief structure of a commercial reef has been established, the question becomes to what extent will increased levels of exploitation impair oyster population productivity and health? In this study, we have directly evaluated the impact of commercial dredging on oyster population productivity and health on an oyster reef that has, over the years, been one of the most significant commercial producers of oysters in Delaware Bay.

METHODS

Field Program

Two pairs of sites were chosen on New Beds, one of the larger seed beds in Delaware Bay (Fig. 1). Both pairs were located in areas where long-term records consistently showed above-average oyster production. Each site was a rectangle of about 0.2 min longitude by 0.2 min latitude. The two site pairs were separated by 0.2 min latitude. The two sites in each pair abutted each other on one of the four sides (Fig. 1). The two sites in a pair were randomly designated as either an experimental site or a control site. These sites were, for the controls, C1 (grid 2) and C2 (grid 37), and for

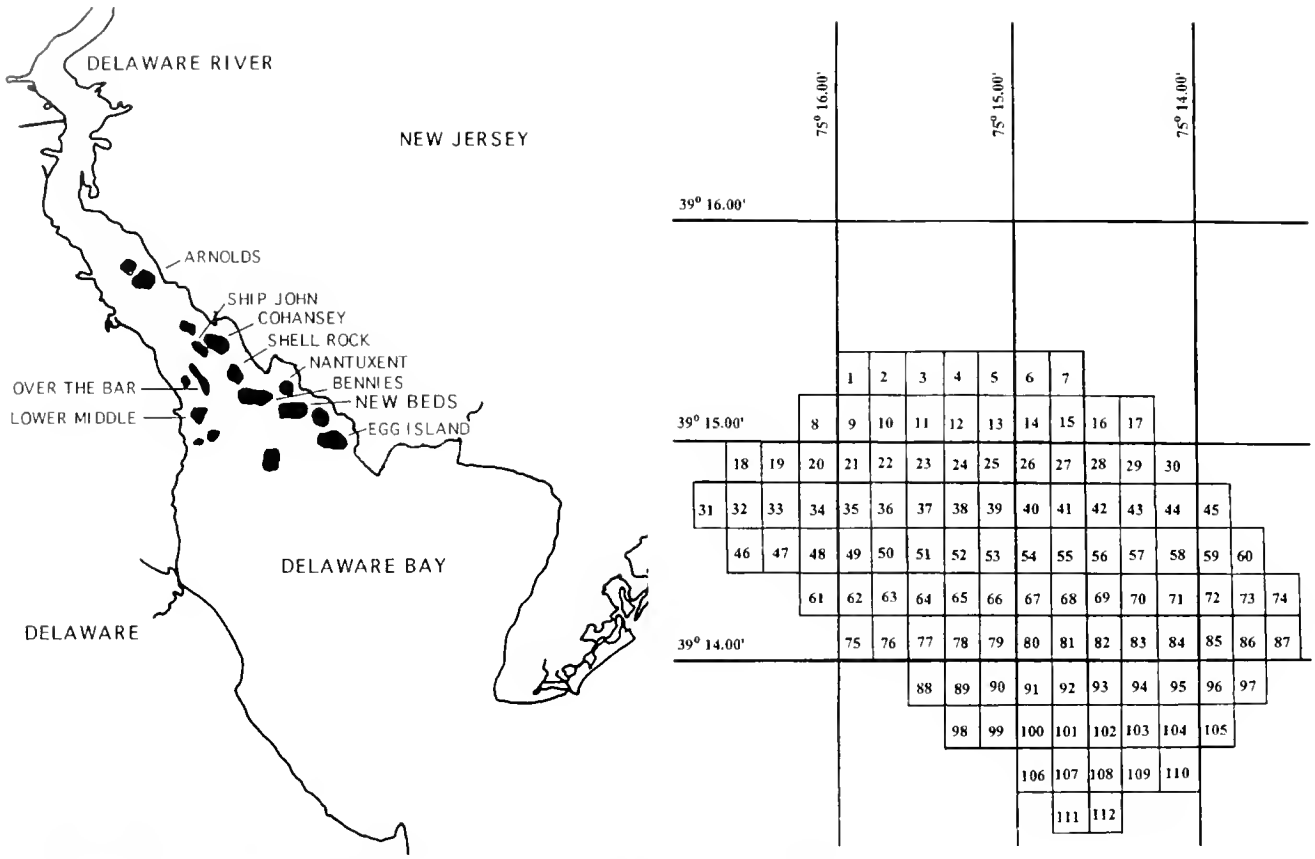

Figure 1. Map of Delaware Bay showing the location of New Beds where the study was conducted and the position of the four sites, grids 2, 10, 36, and 37, on New Beds.

the experimentals, E1 (grid 10) and E2 (grid 36) (Fig. 1). (Fegley et al. [1994] describe the bed gridding system used in Delaware Bay.) Dredging was conducted on the experimental sites.

The four sites were sampled in late October 1999, prior to initiation of dredging. Subsequently, dredging occurred on each experimental site in late October 1999, early and late November 1999, April 2000, and July 2000. Samples were taken from each site prior to dredging, except in November when samples were taken after dredging. The sites were sampled one last time in August 2000.

Between dredging events, fishing should not have occurred on any of the sites. Each site was designated as closed to fishing by the New Jersey Department of Environmental Protection (NJDEP). All of New Beds was closed from Fall 1999 into Spring 2000, thus limiting any chance of fishing unknown to us during most of the experiment. New Beds reopened in May 2000 so that some dredging unknown to us might have occurred on the sites during Summer 2000; however, the sites were buoyed and the NJDEP checked the sites routinely so that any impact of the fishery during that time was certainly minimal in comparison to our efforts.

Dredging was conducted according to standard industry procedures. In the Delaware Bay fishery, some boats carry two dredges, operated port and starboard, and some boats carry one dredge, typically deployed aft. The standard dredge used by nearly all boats has a mouth opening of 1.27 m. Accordingly, we used both a one-dredge and a two-dredge boat, alternating between the two from one dredging event to another, and we used a 1.27-m dredge. Each day, the boat arrived on site around 7:30 AM and ceased fishing around 3:30 PM. Dredging was continuous during the 8-h period. Market-size oysters were culled and bushels were sacked in the standard manner. A New Jersey bushel is about 37 L.

Three one-bushel samples were taken for analysis on the last three dredge hauls of the day in 1999 and prior to dredging in 2000 on the experimental sites and on three randomly selected locations on the control sites each time the experimental site in the pair was sampled. As the tows in the experimental site were taken during the normal course of fishing, they were taken quasi-randomly in that portion of the experimental area receiving the heaviest dredging impact. To maintain consistency, tows in the control area were taken in the same manner (time, distance, etc.). During dredging and sampling, a data logger recorded DGPS position and time at 5-sec intervals. The log was annotated each time a dredge was released or retrieved and each time a sack of oysters was filled.

Laboratory Bushel Sample Analysis

Each bushel sample was sorted into live oysters, boxes (dead, articulated valves), shell, and debris, and the respective volumes were measured. The assumption was that boxes represented recent mortality (Christmas et al. 1997). During sorting, the presence of selected fouling organisms was recorded as absent, present, or abundant. These included the red-beard sponge *Microciona*, the boring sponge *Cliona*, the sabellariid polychaete *Sabellaria*, and serpulid polychaetes. Oyster drills, *Urosalpinx* and *Eupleura*, were counted. The presence of drill egg masses and the flatworm *Stylochus* were recorded. Mud crabs were evaluated as absent, present

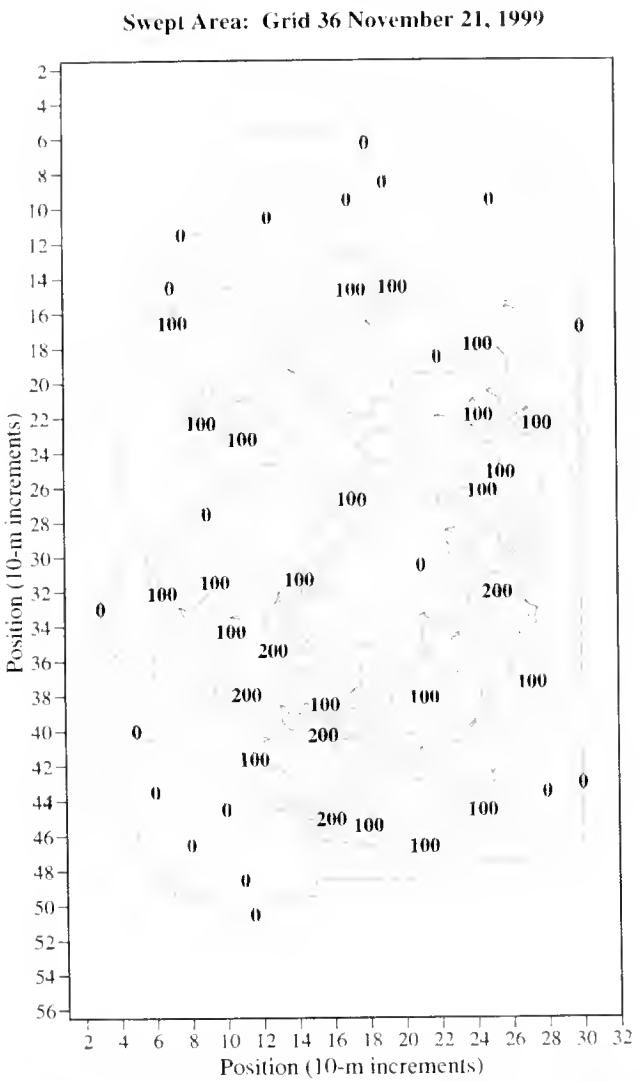

Figure 2. Coverage map of grid 36 (one of the two experimental sites) for an 8-h dredging event that occurred on November 21, 1999. Position is identified on the *x* and *y* axes as the number of 10-m intervals with the (0,0) location in the northwest corner. Contours are the total swept areas dredged in each 10 × 10-m section. Contours were generated from the total estimated coverage for each 10 × 10-m square.

or abundant. The longest dimension of each oyster and box >20 mm was measured. The number of live and dead spat <20 mm was counted. Wet meat weights were taken for each live oyster. At the same time, a random sample of 10 oysters per bushel was selected for *P. marinus* analysis. Mantle and rectal tissue were taken for analysis of infection intensity following standard procedures (Powell & Ellis 1998).

A taphonomic assessment was made for each box and live oyster. The following types of damage were recorded as present or absent on each individual: minor chipping on the shell margin, major chipping on the shell margin, presence of perforations through which tissue could be observed or, for boxes, through which the inside of the shell could be observed, cracking without breakage, and presence of abrasions. Minor and major chipping were distinguished by assigning to the major category cases where small chips were distributed around most of the shell margin or cases where larger pieces of shell were missing. Abrasions were apparent dredge drag marks or cases where the surface of the shell had broken off. Typically, the latter occured on shell heavily bored by clionid sponges. For more information on approaches to taphonomic analysis, we refer the reader to Davies et al. (1990).

Swept Area and Catch per Unit Effort (CPUE) Analysis

Swept area was calculated for each dredge tow from the 5-sec position logs and the dredge width. Site coverage was estimated by dividing the site into 10 × 10-m squares, 100 m^2 in area, and calculating the total swept area in each of these squares. The choice of a 10 × 10-m square was made primarily for ease of data analysis and presentation; however, the dimensions were considered to be the smallest areal dimension that was distinctly larger than the accuracy for which dredge position was known. Dredge position was judged to be accurate within 5 to 10 m. Accuracy was

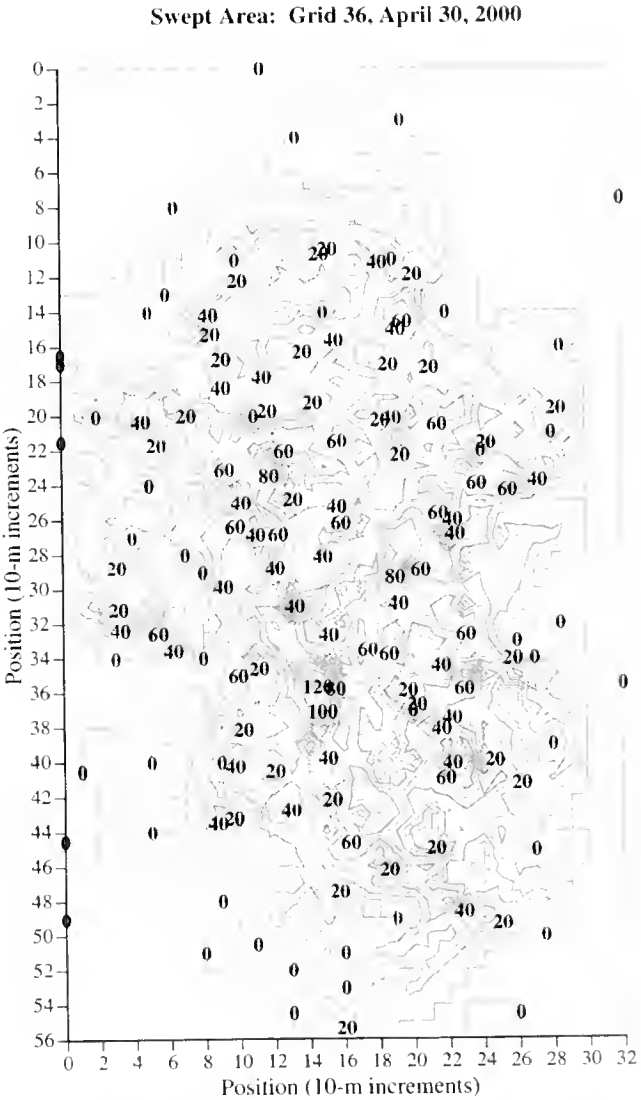

Figure 3. Coverage map of grid 36 (one of the two experimental sites) for an 8-h dredging event that occurred on April 30, 2000. Position is identified on the *x* and *y* axes as the number of 10-m intervals with the (0,0) location in the northwest corner. Contours are the total swept areas dredged in each 10 × 10-m section. Contours were generated from the total estimated coverage for each 10 × 10-m square.

greater for the two-dredge boat because the dredge was released port and starboard forward of amidships, whereas the single-dredge boat released the dredge from the stern. Coverage was assumed to be 100% if the total swept area in a 10 × 10-m square during a single daily event reached 100 m^2.

Samples for analysis were obtained from typical dredge tows. A typical dredge tow covered about 300 to 600 m^2 and intersected 40 to 45 10 × 10-m squares. Individual 10 × 10-m squares were not sampled for this report.

CPUE was calculated as the number of bushel sacks per square meter of dredged area. The number of market-size oysters per sack averaged 318.

Statistical Analysis

Most statistical analyses used ANOVA with all interaction terms. Block (the site pairs), treatment (experimentals and controls), and in some cases, time were class variables. For time trends in CPUE, a Spearman's rank correlation was used. For ANOVA analyses, data on number and volume were normalized to the total volume of shell per bushel (the sum of the volumes of live oysters, boxes, and shell). This was done because the bushel sample did not come from a known area—the dredge catches much more than one bushel—and the volume of debris including biont overgrowth was not a constant over the study, whereas to a first approximation, the volume of shell was. To further assess the influence of using differing sample sizes, we also conducted analyses on ratios of variables, such as the ratio of live oysters to boxes. Such ratios are not influenced by the volume of sample collected. With the exception of presence-absence data that were recorded as a 0 (absent) or a 1 (present), all variables were ranked prior to analyses. Consequently, all statistical analyses were effectively nonparametric. Finally, analyses using variables that potentially were influenced by size, such as Dermo infection intensity, condition index, etc., included size class as a class-variable covariate (Underwood 1997). Size classes were juvenile (20–63.4 mm), submarket (63.5–76.2 mm), and market (>76.2 mm).

RESULTS

Swept Area Coverage

The one-dredge boat addressed an average of 37,039 m^2 of bottom during a single 8-h day. The two-dredge boat addressed

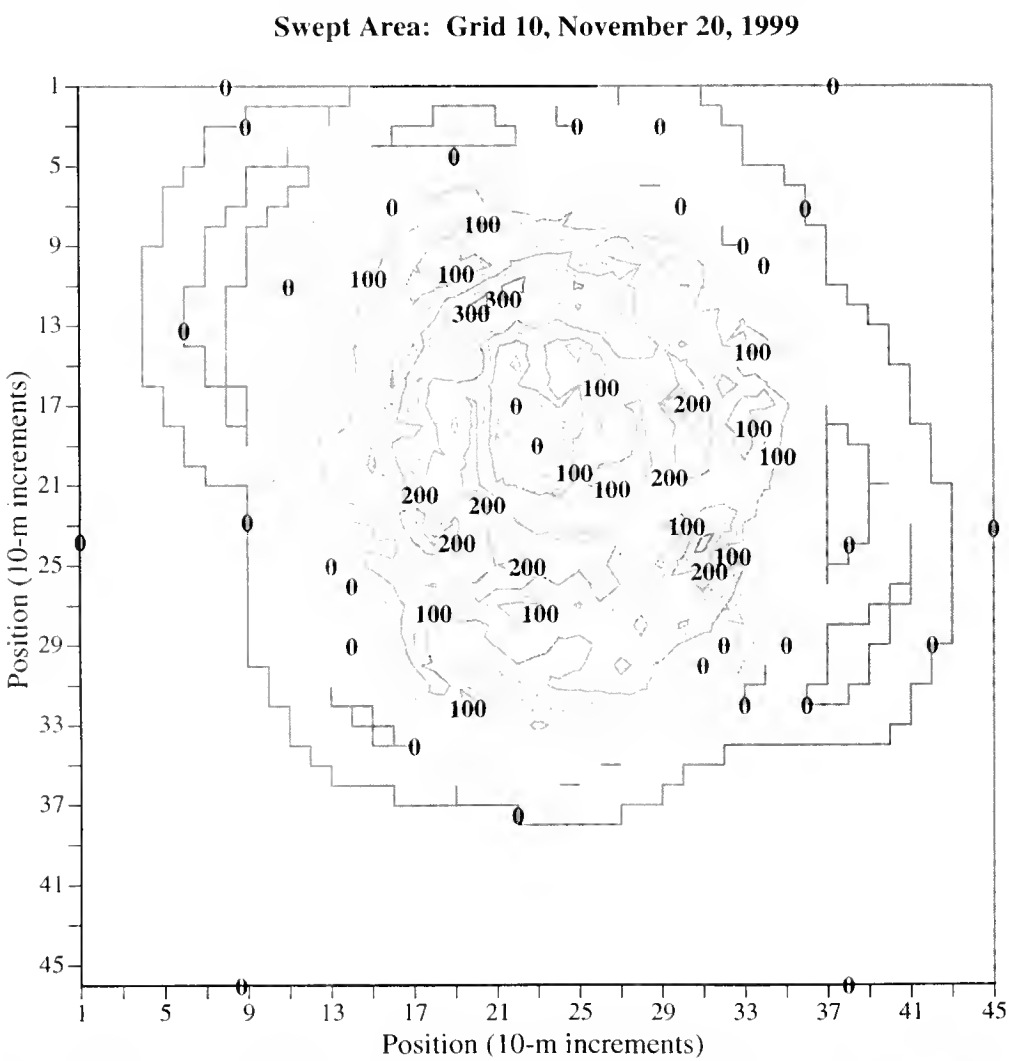

Figure 4. Coverage map of grid 10 (one of the two experimental sites) for an 8-h dredging event that occurred on November 20, 1999. Position is identified on the *x* and *y* axes as the number of 10-m intervals with the (0,0) location in the northwest corner. Contours are the total swept areas dredged in each 10 × 10-m section. Contours were generated from the total estimated coverage for each 10 × 10-m square.

nearly twice that area, 63,606 m^2 on average, because both dredges were on the bottom for much of the day. The pattern of dredging tended to be circular to elliptical around a central point within the 0.2 min latitude by 0.2 min longitude site. Thus, a differential impact occurred over the experimental site, with greatest impact near the epicenter and with the impact steadily decreasing to near-zero at the edge of the site. Daily coverage typically ranged from 50 to >100 m^2 per 10 × 10-m square, meaning that 50% to >100% of a 10 × 10-m square near the epicenter was covered by the dredge during that day. Examples of individual trips are shown in Figures 2 through 5. Total coverage for the study is depicted in Figures 6 and 7. In the heavily dredged central areas of each experimental site, coverage in a 10 × 10-m square varied from 300 to >600 m^2 by the end of the study. That is, an area equivalent to the entire area of a 10 × 10-m square was impacted in that 10 × 10-m square by the dredge three to six times during the study. Although we cannot unequivocally show that each square meter in the 10 × 10-m square was addressed by the dredge three to six times because the position of the dredge on the bottom is not known precisely, certainly we can assume that the entire 10 × 10-m area of such a square was impacted multiple times.

Daily and Cumulative CPUE

CPUE was calculated each time a bushel sack was filled during the day to determine whether the rate of capture declined (Fig. 8). CPUE did not change significantly over the course of the day for any of the 10 dredging events (five 8-h events, two experimental sites; Spearman's rank correlation, $\alpha = 0.05$).

Site Status at Study Inception

All four sites, the two experimental sites and the two control sites, were sampled prior to the initiation of dredging. ANOVA analyses revealed that the experimental sites diverged from the control sites significantly for a few variables, including the amount of fouling ($P = 0.02$), taphonomic status (boxes only, $P = 0.0001$), and condition index ($P = 0.0001$). The significant difference in taphonomic status came from significant differences in

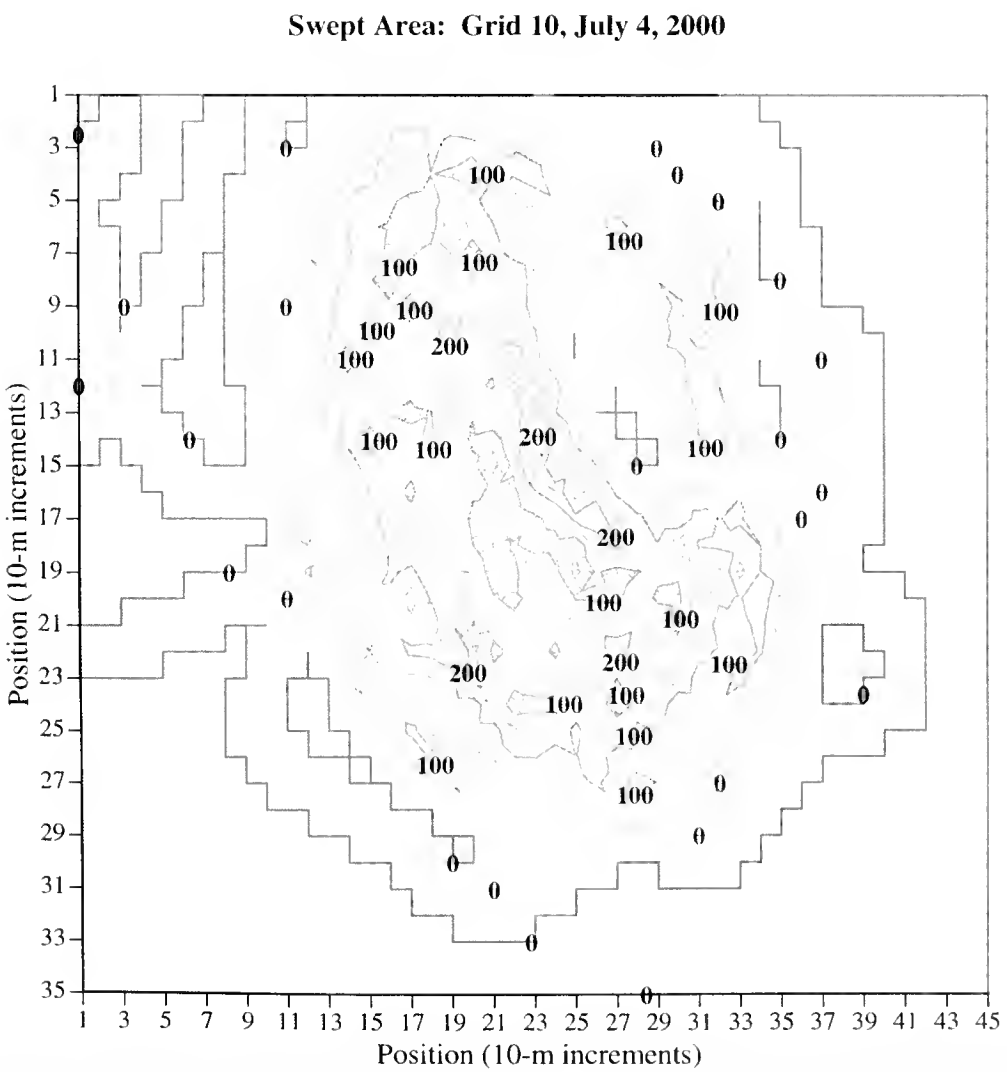

Figure 5. Coverage map of grid 10 (one of the two experimental sites) for an 8-h dredging event that occurred on July 4, 2000. Position is identified on the *x* and *y* axes as the number of 10-m intervals with the (0,0) location in the northwest corner. Contours are the total swept areas dredged in each 10 × 10-m section. Contours were generated from the total estimated coverage for each 10 × 10-m square.

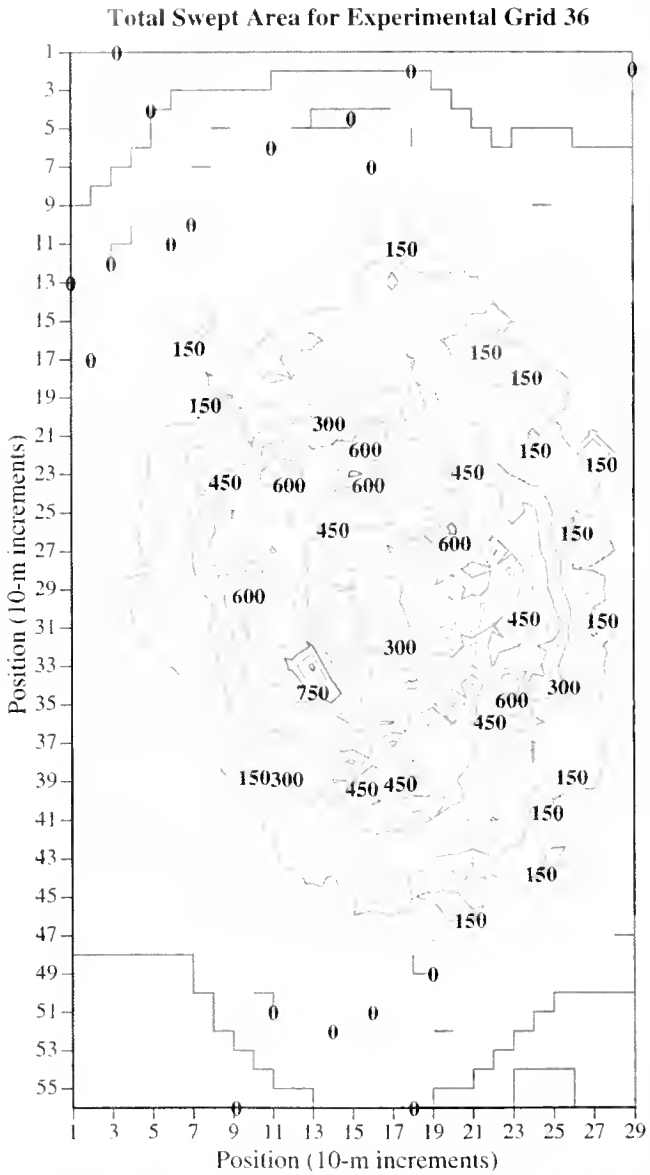

Figure 6. Cumulative coverage map of grid 36 (one of the two experimental sites) for the entire study (five 8-h dredging events = 40 h of dredging). Position is identified on the *x* and *y* axes as the number of 10-m intervals with the (0,0) location in the northwest corner. Contours are the total swept areas dredged in each 10 × 10-m section. Contours were generated from the total estimated coverage for each 10 × 10-m square.

minor chipping and abrasion. Because of the imperfect nature of the controls (e.g., Lindegarth et al. 2000), the evaluation of the effects of dredging will focus on the significance of interaction terms between treatment and time in further analyses.

Immediate Impact of a Dredging Event

The question of the immediate impact of dredging was assessed at the time of the first two dredging events, which took place within 10 days of each other in late October/early November 1999. In this case, the experimental sites were sampled before the first event and after the second.

In only one case was a uniform effect on the two experimental sites found. Minor chipping of live oysters increased after dredging on both experimental sites (Table 1). In general, the incidence of dredge damage increased in live oysters and boxes after dredging (Table 1). In general, the increase in dredge damage was similar for boxes and live oysters. However, 16 h of dredging did not result in significant changes in oyster health as measured by mortality, *P. marinus* infection intensity, or condition index. Such a result might be expected, however, because the samples were taken immediately after the second 8 h of dredging had been completed.

Influence of Coverage

Coverage, defined as the total swept area for the dredge, increased at each experimental site with each dredging event in the time series (Fig. 9). This increase was not uniform over the experimental site, however. Dredging tended to be heavier in some portions of the site than in others (Figs. 2–5). As a result, when a sample was taken, the coverage for the specific area sampled differed, sometimes considerably, between the three replicate samples. This local variation in coverage within an experimental site might differentially influence each of the three samples taken each time period. Because the market-size oysters are sorted from the total dredge haul onboard the boat while the boat is underway, and the remaining material subsequently redistributed in a quasi-random manner over the site, one might not expect significant relationships to exist between the coverage of any specific set of 10 × 10-m squares and the status of the oysters, boxes, and shell located there after dredging had ceased. This is particularly true in our case where any one sampling tow intersected 40 to 45 10 × 10-m squares over the 300 to 600 m distance of the tow. Nevertheless, if such a relationship did exist, coverage of necessity would have to be included in the statistical analysis as a covariate. Accordingly, we examined the degree to which the three samples at a given site and sampling period differed as a function of the coverage recorded for the specific area of the site from which each sample was taken.

Instances where differences in coverage between the three samples significantly impacted a variable within each site and sampling time did not occur more often than expected by chance (binomial test, $\alpha = 0.05$), as anticipated from the dredging and onboard culling process and the length of the sampling tows. Accordingly, coverage was not included as a covariate in further analyses.

Cumulative Impact of Dredging

As time passed and more dredging events occurred on the experimental sites, the difference between the control sites and experimental sites might be expected to diverge. Such variance would be evidence of long-term impacts of dredging. Accordingly, we examined the time series for significant interaction terms between time and treatment. Because we anticipated that the similarity of the control and experimental sites should decrease over time as dredging impact accumulated on the experimental sites, we also examined the level of significance achieved in comparisons of control and experimental sites at each individual time period when a significant "time*treatment" interaction occurred. We expected the difference between control and experimental sites to reach increasingly higher levels of significance as time passed and the cumulative impact of the dredge increased.

Changes in taphonomic status can be expected to occur as

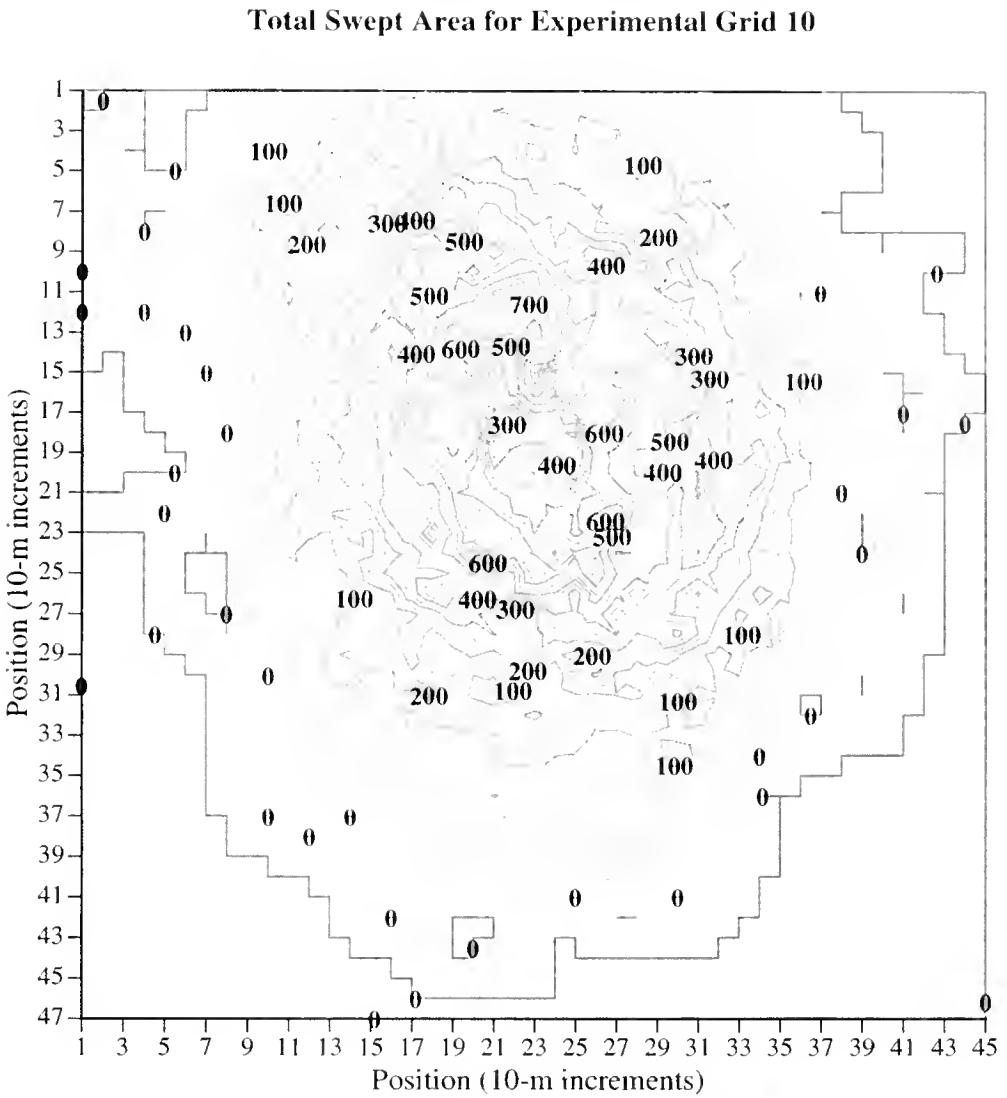

Figure 7. Cumulative coverage map of grid 10 (one of the two experimental sites) for the entire study (five 8-h dredging events = 40 h of dredging). Position is identified on the *x* and *y* axes as the number of 10-m intervals with the (0,0) location in the northwest corner. Contours are the total swept areas dredged in each 10 × 10-m section. Contours were generated from the total estimated coverage for each 10 × 10-m square.

dredging occurs. Indeed, the time*treatment interaction term was significant for total taphonomic impact and for many of the individual taphonomic indicators for live oysters and for boxes (Table 2). However, a consistent pattern over time did not occur. Oysters from experimental sites were more heavily damaged in November and July and less heavily damaged in April, for example (Table 2, Fig. 10). Minor chipping rose slightly over the winter and declined into the summer, presumably as the oysters were able to add new shell, thereby removing the evidence of old chips (Fig. 11). Oysters from the control sites and experimental sites followed very similar patterns in that the incidence of minor chipping declined simultaneously in both. Similarly, abrasive wear rose throughout the study for oysters from both the experimental and control sites (Fig. 12). Oysters from the experimental and control sites differed significantly in the incidence of abrasion in late November, April, and July, but the relationship was inconsistent over time. Abrasion was more common in oysters from control sites than from experimental sites in April and was less common at the other two times. More severe forms of shell damage such as breakage, cracking, and shell perforation occurred rarely (Fig. 13), and were not statistically related to the presence or absence of dredging.

Boxes followed a similar pattern. Boxes from the control sites diverged from the experimentals at the beginning of the experiment, but subsequently did not differ much (Fig. 10). The instance of minor chipping declined in the summer (Fig. 11). The frequency of abrasion rose over the course of the experiment, though less regularly than for live oysters, and boxes from experimental sites diverged from the control boxes only at the inception of the study (Fig. 12). Not surprisingly, considering the similarity of the boxes and live oysters throughout the study, the ratio between the two for the various taphonomic indicators varied little (Table 2, Fig. 14).

Oysters from the control and experimental sites differed little beyond the taphonomic indicators of dredge damage. Time*treatment interaction terms were not significant for the ratio of live oysters to boxes, indicating that the pattern of mortality did not change between control and experimental sites (Fig. 15). The ratio

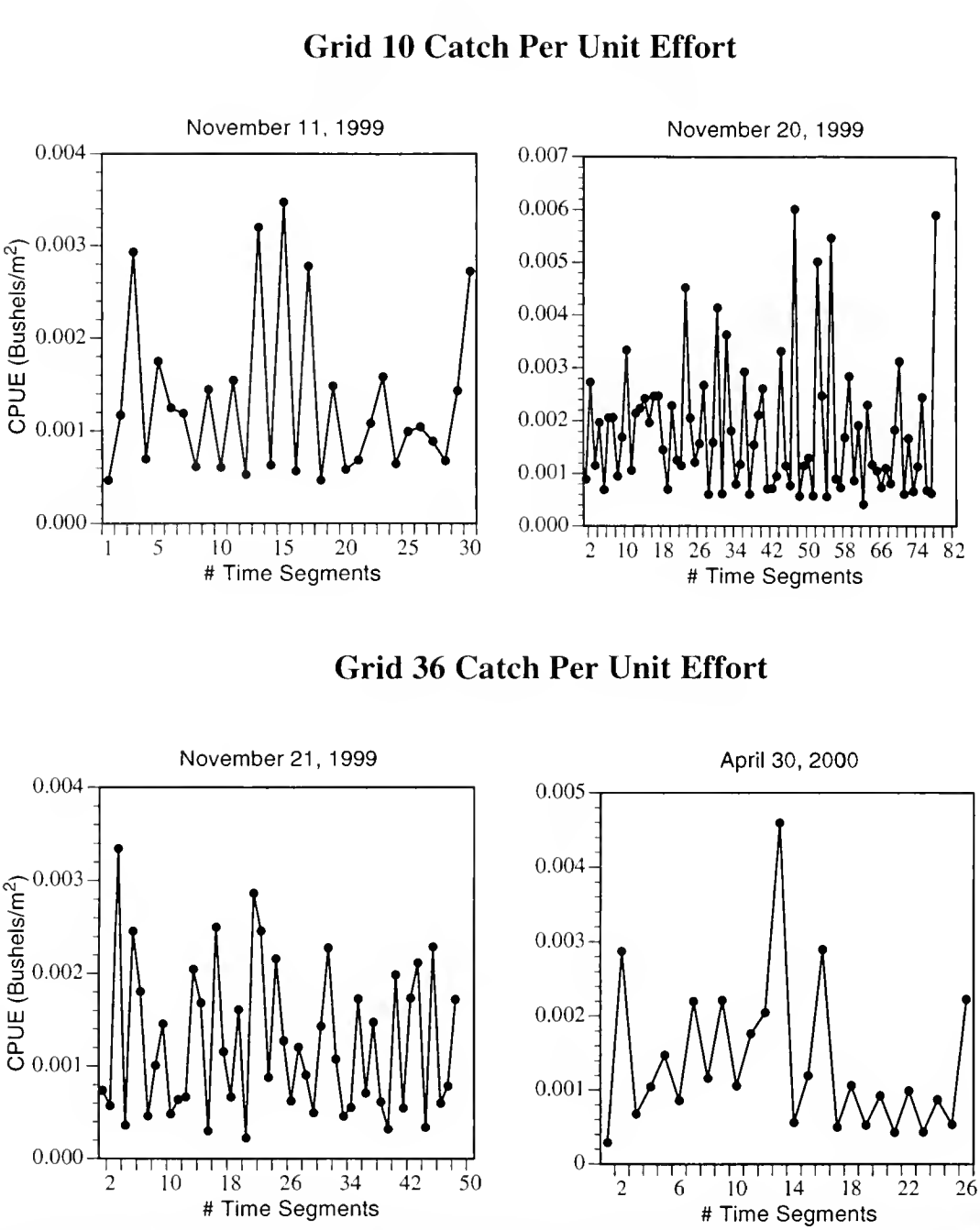

Figure 8. Examples of time trends in CPUE for daily dredging events. CPUE is measured in bushels caught per square meter of swept area. The *x* axis records consecutive time segments when one or more bushel sacks were filled. Each CPUE estimate is based on the swept area during that time segment.

of juveniles to submarkets and submarkets to market-size oysters also did not change, indicating that the processes of growth and mortality changed in similar ways in oysters from control and experimental sites. The time*treatment interaction was significant for condition index, but this result was principally due to the divergence of the experimental and control oysters at the beginning of the study (Table 2). Subsequently, condition index declined slightly over winter in both treatments, rose somewhat into the summer, and then declined again, as is typically the case in Delaware Bay (Fig. 16). Thus, the experimental and control sites yielded very similar values of condition index throughout the study.

DISCUSSION

Coverage and Short-Term Impacts

Total dredge coverage for the study was about 240,000 m^2 on each experimental site (Fig. 9). The most heavily dredged areas were completely covered by the dredge four to six times during the study. This degree of impact is somewhat greater than is typical for the Delaware Bay seed beds under the direct-market harvesting scenario that exists today. Estimates of swept-area coverage in 2000 by industry vessels during the fishing season range from about 50% to 800% of bed area (the latter indicating that the bed

TABLE 1.

Results of ANOVA tests on time for the first two dredging events that took place within 10 days of each other in late October/early November, 1999, in which the condition of the site before and immediately after 16 h of dredging was compared. *P* values under the time and time*site columns record the results of ANOVA analysis for the variable time (before vs. after dredging) and the interaction term with site (the two experimental sites). The column "?Site" records which of the two sites, E1 or E2, were significantly different. The directional arrows indicate whether dredging resulted in an increase (up) or decrease (down) in the variable. Only taphonomic indicators showing significant results are shown. NS, not significant ($\alpha = 0.05$).

Variable	Time	Time*Site	?Site	Direction
Total live oysters	NS	NS		
Total boxes	NS	NS		
Live/Dead ratio	0.03	NS	E2	↑
Number drills	NS	NS		
Fouling status	NS	NS		
Number live spat	NS	NS		
Number dead spat	NS	0.04	E2	↑
Condition index	NS	0.0003	E2	↓
Perkinsus marinus infection intensity	NS	NS		
Taphonomic status, live	0.0001	0.02	E2	↑
Minor chipping	0.0001	NS	E1, E2	↑
Abrasion	0.005	NS	E2	↑
Shell perforation	0.02	NS	E2	↓
Taphonomic status, dead	0.0001	0.0004	E2	↑
Minor chipping	0.004	NS	E1	↑
Major chipping	0.02	NS	E1	↓
Abrasion	0.0001	0.0007	E2	↑
Taphonomic status, ratio live/dead	NS	NS		

was completely dredged eight times in 2000, depending on the seed bed, but only one bed exceeded 150% of bed area (1.5 times; Banta et al. unpubl. data). Average coverage for the fished oyster beds in 2000 was 84%. Thus, our study produced dredge swept-area coverages of approximately double to triple the oyster fishery's impact on an average heavily fished oyster bed in Delaware Bay. It follows that any impacts recorded in this study are likely, on the average, to be more severe than typically observed under normal fishing activities in Delaware Bay.

Sixteen hours of dredging, two 8-h events within a 10-day period at the beginning of the study, produced barely detectable changes in the oyster population. Given the rigorous nature of dredging, one might anticipate that shell cracking and breakage would occur often enough to be readily detected. In fact, these severe forms of impact occurred rarely. The incidence of cracking and breakage did not normally exceed about 2% of the live oysters, and major chipping was restricted to about 20% of the population. These more serious forms of shell damage did not increase in frequency after 16 h of dredging. Minor chipping and abrasion were more common, about 70% of the oyster population had minor chipping and a more variable number, 10% to 50%, showed evidence of abrasion. These latter two less serious forms of dredge damage did increase in frequency after a 16-h period of dredging. However, no other discernible impacts were found.

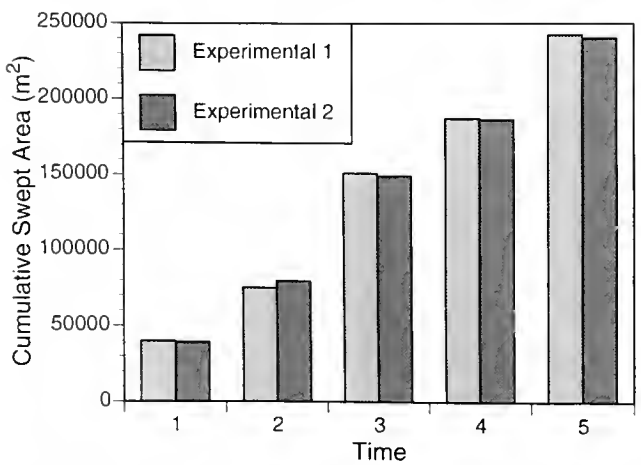

Figure 9. Cumulative swept-area coverage for the two experimental sites. Time segments are defined in Figure 10.

Long-Term Impact: Dredge Damage

Dredge coverage accumulated at an average of about 47,500 m^2 per 8-h event on each experimental site (Fig. 9). Although the impact of a single event was barely measurable, dredge damage might be expected to accumulate over the study. No such trend was observed.

Many of the taphonomic indicators of dredge damage showed time-dependent trends that differed between control and experimental sites. However, for the most part, these effects were limited to minor chipping and indications of abrasive wear, rather than the more serious aspects of shell damage defined as major chipping, breakage, cracking, and shell perforation. Furthermore, the anticipated divergence between control and experimental sites was most noticeable on the sampling dates that immediately preceded and followed the winter months. By summer, most differences had disappeared.

Two possible reasons exist for the failure of dredge damage to accumulate over the study as might have been anticipated. (1) The taphonomic indicators might represent damage that occurs from sources other than dredging. Certainly, chipping and breakage can be predator induced, for example (McDermott 1960, LaBarbera

TABLE 2.

Results of ANOVA tests on time for the full time series of dredging events. *P* values under time*treatment, time*treatment*block, time*treatment*size class, and time*treatment*block*size class record the results of ANOVA analysis for interaction terms with time and treatment. *P* values given under the Time columns (1, and 3–6) represent significant treatment effects obtained from ANOVA analysis for that time only. These times correspond to those given in Figures 10–15. The absence of Time 2 in the table results from the absence of control samples at that time (this was the second sampling of the experimental site taken immediately after the first two dredging events). Only taphonomic indicators showing significant results are shown. NS, not significant ($\alpha = 0.05$). –, not applicable.

Variable	Time*Treatment	Treatment* Time*Block	Treatment* Time*Size Class	Treatment*Block* Time*Size Class	Time 1	Time 3	Time 4	Time 5	Time 6
Total live oysters	NS	NS	–	–					
Total boxes	NS	NS	–	–					
Live/Dead ratio	NS	NS	NS	NS					
Number drills	NS	NS	–	–					
Fouling status	0.04	NS	–	–					
Number live spat	NS	NS	–	–					
Number dead spat	NS	NS	–	–					
Condition index	0.004	NS	NS	NS	0.0001	NS	NS	0.005	NS
Perkinsus marinus infection intensity	NS	0.03	NS	0.04	NS	NS	NS	NS	NS
Juvenile/Submarket ratio	NS	NS	–	–					
Submarket/Market ratio	NS	NS	–	–					
Taphonomic status, live	0.0001	NS	NS	NS	0.01	0.001	0.003	0.0006	NS
Minor chipping	0.001	NS	NS	NS	NS	NS	0.004	NS	NS
Breakage	0.02	NS	NS	NS	0.03	NS	NS	NS	NS
Abrasion	0.001	NS	NS	NS	NS	0.001	0.004	0.008	NS
Taphonomic status, dead	0.0001	0.05	0.02	NS	0.0001	0.03	NS	NS	NS
Minor chipping	0.002	NS	NS	NS	0.02	0.02	0.01	NS	NS
Breakage	0.006	NS	0.03	NS	NS	0.02	0.03	NS	NS
Abrasion	0.0001	0.0001	0.0001	NS	0.0001	NS	NS	NS	NS
Taphonomic status, ratio live/dead	NS	NS	NS	NS					
Minor chipping	0.009	NS	NS	NS	NS	NS	0.008	NS	NS
Breakage	0.007	NS	0.03	NS	0.03	0.02	NS	NS	NS

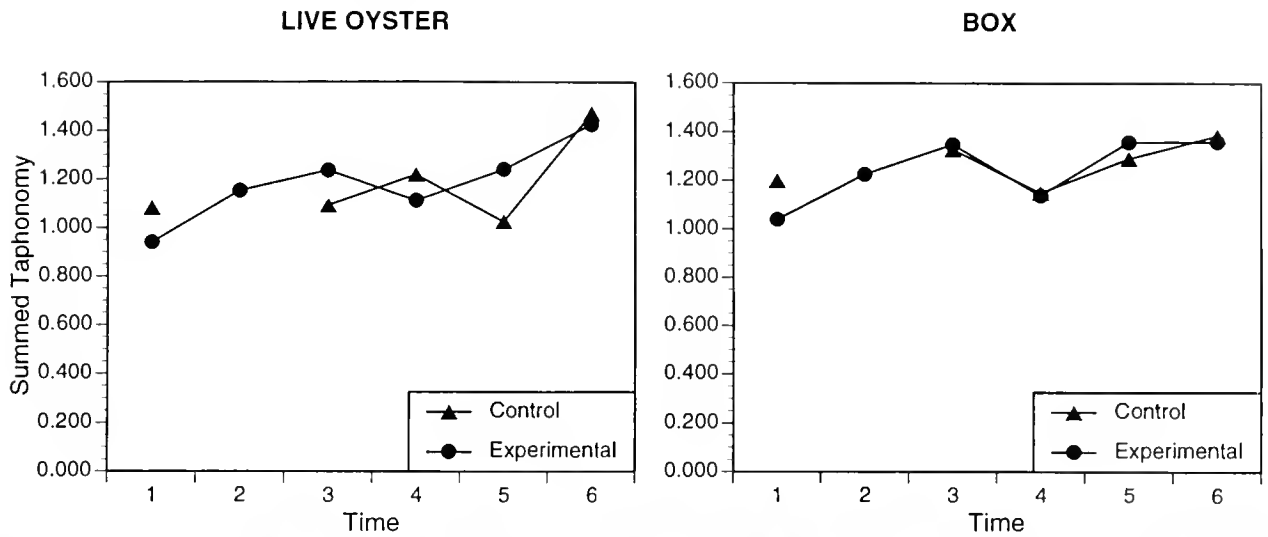

Figure 10. Time-dependent changes in the taphonomic condition of live oysters and boxes during the study. Taphonomic condition is calculated as the sum of minor chipping, major chipping, abrasion, breakage, cracking, and shell perforation. For example, if two of these conditions were present, the sum recorded would take the value of 2. Time segments are: 1, October 30–31, 1999; 2, November 11–13, 1999; 3, November 20–21, 1999; 4, April 28–30, 2000; 5, July 2–4, 2000; 6, August 31, 2000.

1981, Elner & Lavoie 1983). (2) Rapid repair of minor shell damage may mitigate the dredging impact. Repair should occur most rapidly during the warmer months and, in fact, the incidence of minor chipping decreased substantially during the summer. This decrease suggests that rapid shell repair during the warmer months is an important mechanism minimizing the observable impact of dredging, although some effect on the animal's energy budget, in diverting energy to shell growth, might be anticipated (Bernard 1974).

The incidence of abrasive wear increased throughout the study on both sites (Fig. 12). Abrasion on the shell surface cannot be repaired and so would be expected to accumulate. However, the fact that this taphonomic indicator increased in both the experimental and control oysters suggests that its origin is something other than dredge damage. Increased clionid activity was noted during the study (Fig. 17) and would explain this observation.

Juvenile, submarket, and market-size oysters might be expected to differ in their sensitivity to shell damage by dredging. No evidence could be obtained for a differential effect in this study, however.

Long-Term Impact: Population Health

One assumes that oysters, like other bivalves, respond at the physiological level to the stress of capture and release (Pekkarinen & Suoranta 1995). No physiological indices of stress were evaluated in this study. However, a variety of population health indicators were assayed during the study that should integrate changes in underlying physiological state via measures of growth, disease pressure, and mortality. These variables included the ratio of live oysters to boxes, condition index, *P. marinus* infection intensity, and ratios between oyster size classes.

Essentially no significant effects could be discerned for any of these measures. The ratio of live oysters to boxes remained relatively similar between control and experimental sites (Fig. 15). Even when the oyster and box populations were apportioned into three size classes, the ratio of live oysters to boxes was not influ-

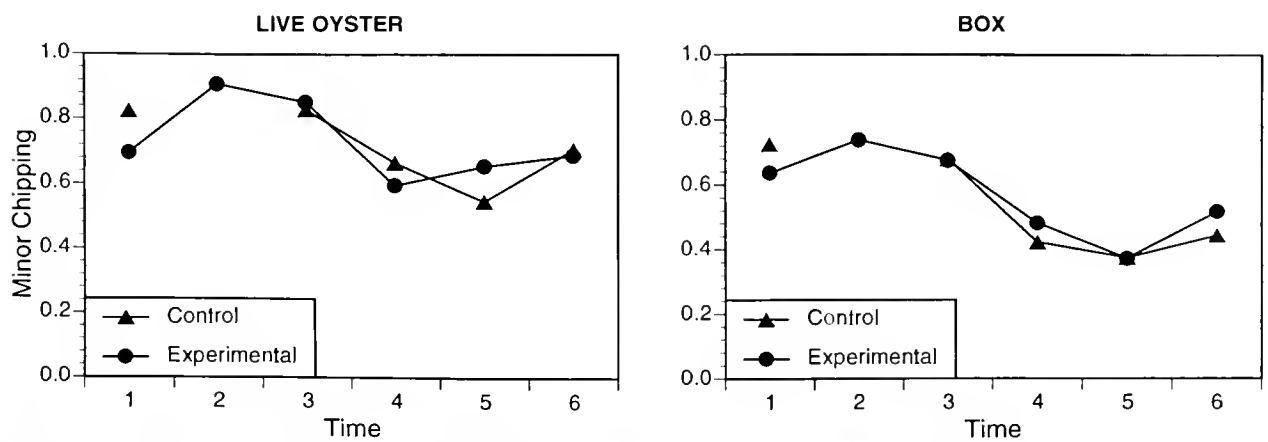

Figure 11. Time-dependent changes in the the frequency of minor chipping of live oysters and boxes during the study. Time segments are defined in Figure 10.

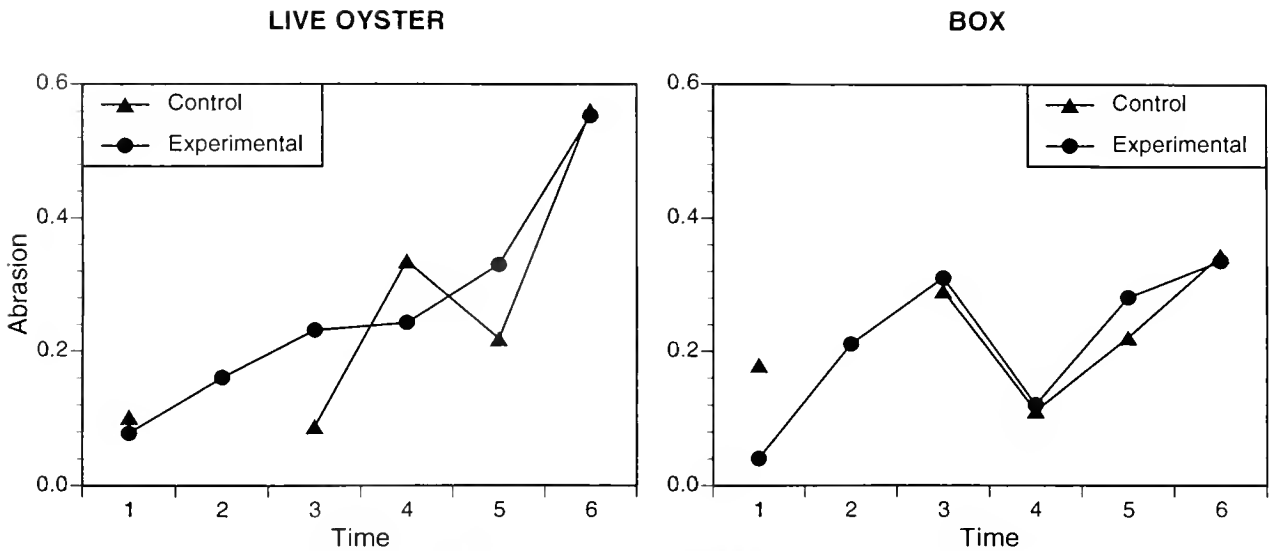

Figure 12. Time-dependent changes in the frequency of abrasion of live oysters and boxes during the study. Time segments are defined in Figure 10.

enced significantly by repeated dredging. Thus, certain size classes were not differentially affected. The ratio of juvenile to submarket oysters and submarket to market-size oysters similarly did not diverge significantly over time between the control and experimental sites. *P. marinus* infection intensity was also unaffected. Dredging, in the degree conducted in this study, did not measurably impair oyster growth, mortality, or population health.

Because the fishing effort resulted in the removal of market-size oysters from the experimental sites, the ratio of submarket to market-size oysters in the experimental and control sites might have been expected to diverge over time. This did not occur because the industry dredge efficiency averaged between 4% and 7% (Banta et al. unpubl. data). Thus, despite the rigorous dredging activity conducted during the study, no more than approximately 25% of the market-size oysters were removed. This low removal rate and the likely growth of some submarket-size oysters into market size during the study prevented a measurable reduction in market-size oysters from taking place.

One result of the low dredge efficiency during fishing operations is the constant redistribution of shell and oysters without retrieval to the fishing vessel. Presumably, this redistribution should result in burial of some portion of the oyster population for some period of time. Oysters are well known for being able to withstand prolonged periods of anoxia that might occur with burial (Shumway 1982, Baker & Mann 1992), however, the time between dredging events was typically several months. Thus, an increase in mortality due to burial might have been anticipated. None was observed, indicating that the redistribution of shell and oysters must normally not result in burial so deep that oysters cannot open their valves and filter at adequate rates.

Increased recruitment is often observed on planted shell (Abbe 1988). In the same vein, the activity of fishing by turning over shell and exposing clean surfaces might increase recruitment. Our study was done during a period of low recruitment on the lower half of the Delaware Bay oyster seed beds, including New Beds, and so the results may not be generally applicable. However, re-

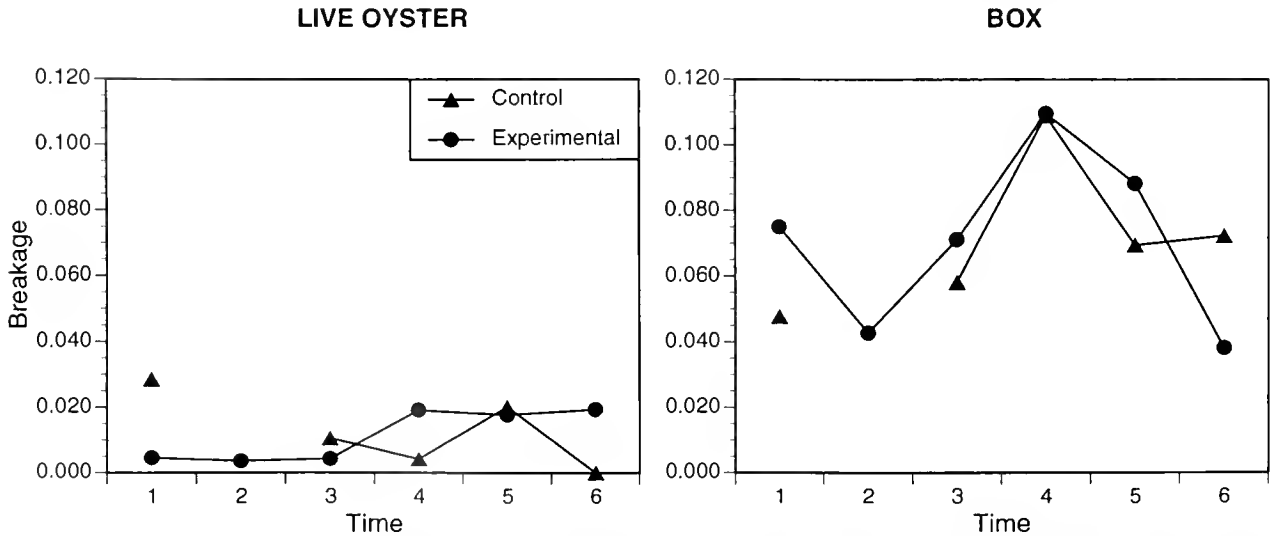

Figure 13. Time-dependent changes in the frequency of breakage of live oysters and boxes during the study. Time segments are defined in Figure 10.

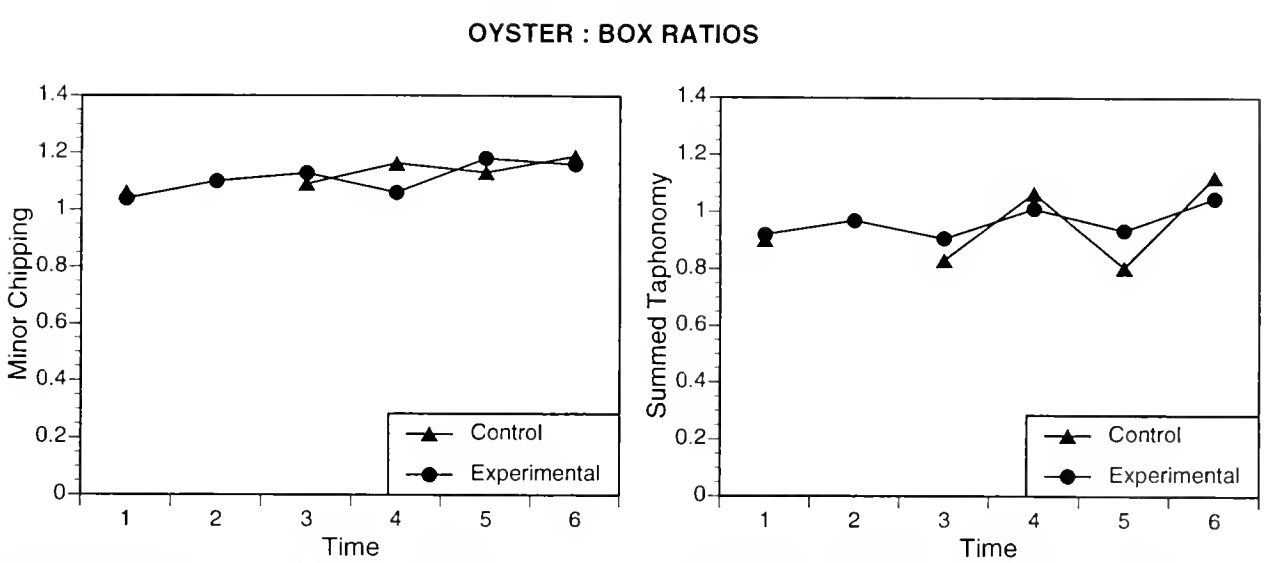

Figure 14. Time-dependent changes in the ratio of selected taphonomic indicators between live oysters and boxes during the study. The ratio is calculated as $\frac{\text{indicator for live oysters}}{\text{indicator for boxes}}$. Time segments are defined in Figure 10.

peated dredging in this study did not significantly affect spat recruitment or mortality.

Taphonomic Indicators: Boxes vs. Live Oysters

The taphonomic indicators consistently showed a surprising similarity between boxes and live oysters. Ratios between live oysters and boxes for the various taphonomic indicators were normally 1 ± 0.1 (Fig. 14). Thus, seasonal changes in taphonomic condition of live oysters were followed reasonably well by seasonal changes in taphonomic condition of boxes. The implication is that most boxes are not very old, probably less than six mo. Christmas et al. (1997) observed boxes routinely surviving for several years in Chesapeake Bay. Our observations would suggest that disarticulation rates are much higher in Delaware Bay.

We observed no significant reduction in box number due to dredging, even though one might assume that dredging should result in some degree of disarticulation. Thus, the apparently short "life span" of boxes does not seem to be a function of fishing. The similarity between control and experimental sites also indicates that disarticulation rates are not significantly increased by fishing activity. The study was conducted during a time when mortality

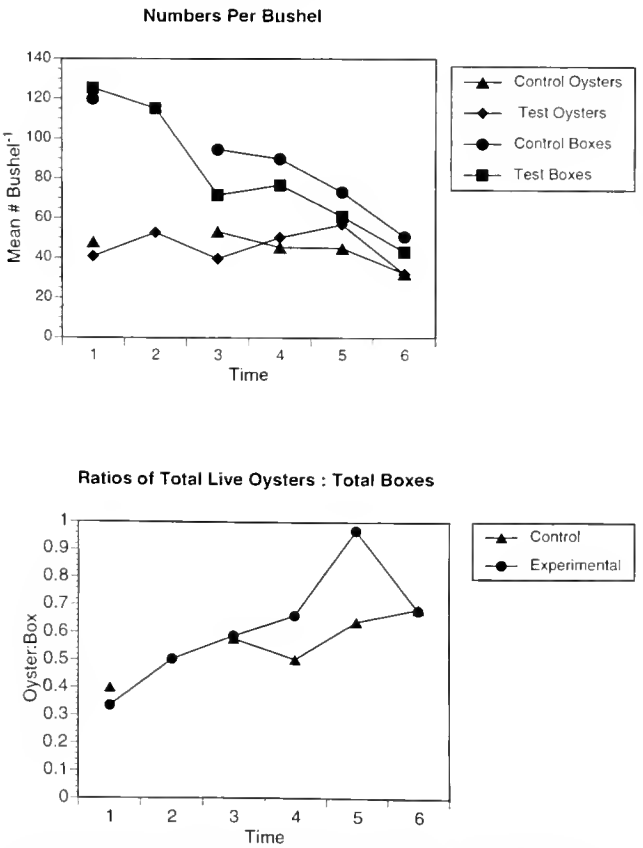

Figure 15. Time-dependent changes in the number of live oysters and boxes per bushel of shell and the ratio of live oysters to boxes during the study. The ratio is calculated as $\frac{\text{number of live oysters}}{\text{number of boxes}}$.

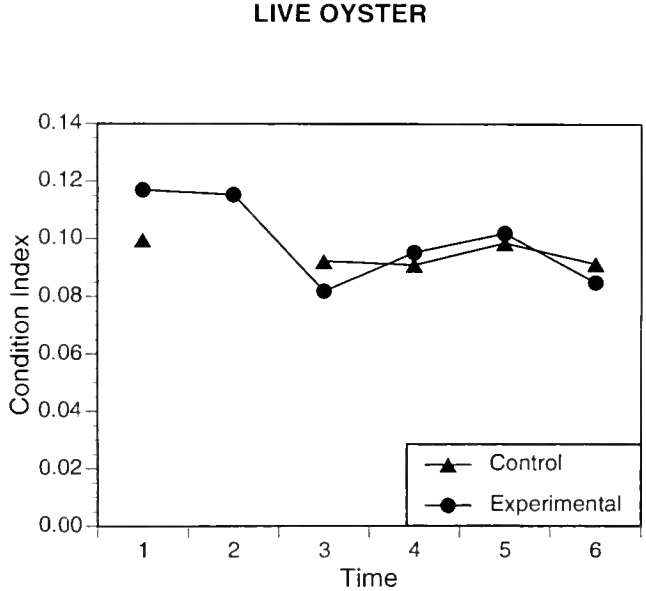

Figure 16. Time-dependent changes in condition index for live oysters during the study. Time segments are defined in Figure 10.

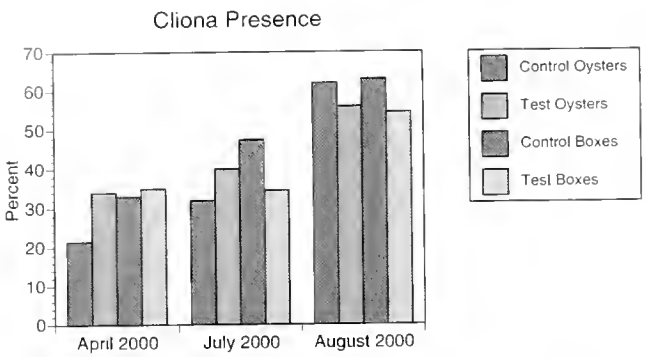

Figure 17. Time-dependent changes in the percentage of oysters and boxes identified as *Cliona* infested during the study. Time segments are defined in Figure 10.

from *P. marinus* was high, greater than 50% of the market size population on New Beds in 2000. Thus, Dermo mortality is the likely principal source of new boxes. Boxes typically outnumbered live oysters by a factor of two at the beginning of the study (Fig. 15). Thus, disarticulation rates must average 25% or more yearly at steady state. In fact, during the study, the number of boxes decreased by a factor of about two (Fig. 15) and the live oyster-to-box ratio increased from about 0.4 to 0.7. Thus, the ongoing mortality in the oyster population imposed an increasingly large signal on the box population as the study progressed. Under these conditions, the taphonomic condition of the box population should always mirror the taphonomic condition of the living oysters to a substantial degree.

Why disarticulation rates were so high is unknown. One possibility is the high clionid activity noted during the study (Fig. 17) that may have weakened the valves of boxes and increased disarticulation rates.

CONCLUSIONS

Over a very long time, dredging may significantly influence oyster bed physiography and community structure. The reduction in the number and size of oyster clumps on fished beds is a good example. However, once the bed has become a fished bed, this study suggests that moderate dredging that results in a yearly swept area of no more than four times the area of the bed is unlikely to result in significant further impact on the oyster populations living there. In this study, no evidence was obtained for increased mortality, decreased growth or recruitment, or increased *P. marinus* disease pressure from repeated dredging. This is a considerably different result from the observed impact of bottom fishing on some soft-bottom bivalves (e.g., Witbaard & Klein 1994) and certainly arises out of the more robust nature of the oyster shell combined with the oyster's relatively rapid capability for shell repair.

A question could be raised as to the degree of impact necessary to effect a measurable change in oyster population attributes. Due to the nonlinearity of most taphonomic processes (Callender et al., in press; Staff et al., in press) and most physiological processes (e.g., Powell et al. 1996, Ford et al. 1999), statistical modeling based on the results of this study is unlikely to provide accurate insight. A study in which cumulative impact was substantially increased over the 300 to 600 m^2 per 10 × 10-m square obtained in this study would be necessary. However, since the cumulative dredging impact in this study already exceeds by a considerable measure the impact of today's fishery in Delaware Bay, our results are sufficient to conclude that dredging at present levels of exploitation is relatively benign in its influence on oyster population health and productivity.

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A FISHERIES MODEL FOR MANAGING THE OYSTER FISHERY DURING TIMES OF DISEASE

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ABSTRACT Setting the yearly allocation for a fished stock is always an uncertain endeavor. Populations suffering significant mortality from disease require particularly careful management. Disease mortality is not a standard component of fisheries models, however. Here, we develop a model for the management of fished oyster populations in which disease mortality is a controlling influence. The model requires a quantitative estimate of abundance by size class, some knowledge about growth rates to establish the size range recruiting into the fishery, and an estimate of the anticipated natural mortality rate. The latter is of considerable consequence because small changes in mortality rate effect large changes in predicted outcomes. The model permits investigation of scenarios that include a range of allocations, timing of fishing seasons, variation in fishing efforts within seasons to establish a preferred harvest level, variations in the distribution of fishing among beds to minimize overharvesting of disease-affected beds (area management), and rebuilding plans to increase total stock abundance after epizootic mortality or periods of overharvesting. The model is sufficiently general that it can be applied to any commercial shellfish species. Simulations show that appropriate timing of the fishing season with respect to the timing of disease mortality can more than double the yearly allocation to the fishery. Some harvested animals would otherwise have died from disease. Besides disease, the other model parameter that most affects simulation outcome is the abundance of submarket-size oysters that can be expected to recruit to the fishery in the simulated year. Population stability is strongly determined by the number of recruits available to replace the deaths that decimate the market-size population each year. The number of recruits is a function of survivorship in previous years, but also the anticipated growth rate that defines the size range of oysters at the beginning of the fishing year that can be expected to recruit to the fishery. This modeling exercise points to the critical need to understand population dynamics and survival of size classes below market size that are not often the targets of investigatory activities.

KEY WORDS: fisheries models, management, mortality rate, disease, Dermo, stock assessment

INTRODUCTION

At one time, Delaware Bay was one of the leading producers of American oysters (*Crassostrea virginica*) in the United States (Mackenzie Jr. 1996, Ford 1997). Like many other East coast estuaries, diseases, principally caused by the protozoan parasites *Haplosporidium nelsoni* and *Perkinsus marinus*, have seriously reduced oyster abundance and, consequently, the oyster industry has declined (Ford 1997, Fegley et al. 1994). The diseases have also necessitated changes in the way oysters are harvested. At one time, oystermen transplanted oysters from upbay seed beds downbay to leased grounds for grow-out. These higher-salinity leased grounds have fallen into disuse or production on them has been seriously curtailed by the high rates of disease mortality of oysters planted there. Consequently, new approaches have been proposed (Ford & Haskin 1988, Ford 1992, Powell et al. 1997). One of these options is to concentrate all phases of production on the upestuary seed beds where lower salinity limits disease-produced mortality, at least to some extent. Concentrating all phases of production on the seed beds necessitates increased vigilance by oyster managers (HSRL, 2000, 2001).

In Delaware Bay, the highest quality oysters are produced naturally on the lower third of the seed beds (Fig. 1). Consequently, in recent years, oyster production has focused on two production schemes: (1) direct marketing of oysters produced on these lower seed beds, essentially a wild fishery; and (2) transplanting oysters downbay to these lower seed beds with subsequent recapture for sale. Upbay oysters tend to be smaller and of lower market quality. Both conditions improve within a few months to a year when moved downbay (Powell et al. 1997). One necessary consequence of both of these approaches is a need to estimate the total allowable catch (TAC) from the seed beds each year, essentially the setting of a yearly allocation for transplant downbay and for direct marketing (HSRL, 2000, 2001).

The problem of setting an allocation is an interesting one because oyster diseases produce high natural mortality rates that are relatively unpredictable from one year to the next (Jordan 1995, Powell et al. 1996, Brousseau et al. 1998). This obviates the possibility managing under standard fisheries guidelines that focus on biomass at maximum sustainable yield (B_{msy}) and normally define B_{msy} as one-half of the carrying capacity ($K/2$) (May et al. 1978, Applegate et al. 1998, Restrepo et al. 1998). Disease mortality is not a standard component of fisheries models. Even in cases where natural mortality may be principally caused by disease, a standard fisheries modeling approach has been used (Allen, 1979). Nor is it clear that K is a concept that can be applied straightforwardly to populations whose dynamics are inherently controlled by disease. Population models addressing the issue of carrying capacity and disease, such as described by Heesterbeek and Roberts (1995) and Swinton and Anderson (1995), have not yet been applied to marine species, although the need for doing so continues to increase (Harvell et al. 1999).

What seems clear is that oyster populations are well below pre-disease levels in many Mid-Atlantic bays today (Andrews 1988, 1996, Gottlieb & Schweighofer 1996, Ford 1997) and that recovery to pre-disease levels is unlikely with the continued presence of disease. The purpose of this contribution is to develop a model for the management of fished oyster populations in which disease mortality is a controlling influence and for which management options cannot be described in terms of B_{msy} and K. We use, as an example, the oyster populations and oyster fishery of Delaware Bay (Fig. 1). Because many of the same issues exist for a

Figure 1. Location of the oyster seed beds in Delaware Bay referred to in this report.

number of commercial shellfish species where disease mortality is significant (e.g., Sousa 1991, Miller & Lawrenz-Miller 1993, Moyer et al. 1993, Park et al. 1999, Bower et al. 1999, Sunila et al. 1999), this modeling approach may have a much wider applicability.

The Model

The modeling exercise begins with the familiar equation

$$\frac{dN}{dt} = -ZN \tag{1}$$

that describes a change in abundance (N) as a function of mortality rate (Z). Equation (1) is solved under the boundary condition that $N = N_0$ at $t = 0$:

$$N(t) = N_0 e^{-Zt} \tag{2}$$

where t is time in days. For a fished species, total mortality rate, Z, can be decomposed into a natural mortality rate, m, and a mortality rate due to fishing, f: $Z = m + f$. Thus,

$$N(t) = N_0 e^{-(m+f)t}. \tag{3}$$

Application of equation (3) to management of the Delaware Bay oyster industry faces a number of serious problems, all of which stem from the time-dependency of the two mortality rates. The first of these problems is that the natural mortality rate, m, is principally determined by Dermo disease. Dermo disease intensifies during the summer months, generally culminating in increased mortality in late summer and fall (Andrews 1988, O'Beirn et al. 1994, Powell et al. 1996, Oliver et al. 1998, Brousseau & Baglivo 2000). Thus, m is not constant over the year. Secondly, the fishery does not impact the oyster population equivalently throughout the year. Typically, a spring and a fall season are separated by a summer period when harvesting is minimal and fishing normally ceases during the winter (HSRL, 2000, 2001). Equation (3) assumes that the two mortality processes, fishing (f) and natural mortality (m), covary such that neither varies seasonally or, if they do vary seasonally, both rise and fall proportionately during the year. This is patently not the case for the natural mortality rate of most estuarine shellfish and rarely the case for fishing mortality for most estuarine shellfisheries. Finally, some portion of fishing mortality is likely to be compensatory because some of the oysters harvested during the spring and early summer would have died from Dermo disease in the following late summer and autumn. Whether different sources of mortality are additive or compensatory is a critical issue in the population dynamics of species where mortality from parasitism and disease is important (Jakobsen et al. 1988, Meißner & Bick 1997, see also Thomas & Kunin 1999). In this case, knowing how the two sources of mortality, from disease and fishing, interact may be particularly important because one of them, fishing, can be manipulated in time to maximize the compensatory component and, consequently, reduce the effect of the fishery on the stock.

A more complex formulation than equation (3) is needed to address issues of seasonality in mortality rates and the interaction between mortality rates. The following model is designed to resolve the issue of time-dependent mortality rates and compensation between mortality rates, while retaining the desirable characteristic of allowing an analytical solution. For simplicity in model construction, we have chosen to define the process of recruitment into the fishery as a rate, like mortality. This requires that the rate of recruitment into the fishery be a function of the number of market-size animals present; however, a standard stock-recruitment relationship (Hilborn & Walters 1992) is rarely, if ever, present (Loosanoff 1966, Hofstetter 1977, Hofmann et al. 1992, Whitlatch & Osman, 1994). In the Delaware Bay fishery, market-size oysters are typically ≥76 mm in length, although no regulatory size limit is in force. Oyster growth rates vary within and between bays along the East and Gulf coasts (O'Beirn et al. 1996, Mackenzie & Wakida-Kusunoki 1997, Dittman et al. 1998). On the direct-market beds of Delaware Bay, growth rates are such that oysters of about 63.5–76 mm in January, defined as submarkets, can be expected to recruit to the fishery in the coming year. Because no regulatory size limit exists and oysters recruit to the fishery in an uneven and unpredictable way (non-knife edge), we resolve the issue of dependency of recruitment on the abundance of market-size oysters by defining recruitment rate such that the recruits reach market size on the first day of the year. This permits the dependency to be artificially defined at the beginning of each simulated year by appropriate choice of recruitment rate (R) with respect to market-size abundance (N).

Accordingly, equation (3) describing the change in abundance over time is expanded to

$$\frac{dN}{dt} = [-Z(t) + R(t)]N \tag{4}$$

where Z represents the sum of a series of time-dependent mortality rates. For the purposes of the present application to management of the oyster fishery in Delaware Bay, Z includes f_1, the fishing rate during the first (spring) season, f_2, the fishing rate during the

second (autumn) season, and m_o the non-fishing mortality rate (mainly Dermo-produced during late summer and early fall). Each of these processes is active over only part of the year. Hence, for market-size oysters

$$\frac{dN}{dt} = [-f_1(t) - f_2(t) - m(t) + R(t)]N. \tag{5}$$

The general solution for this model is

$$N(t) = N_o e^{A(t)}, \tag{6}$$

where N_o is the number of market-size oysters present at the beginning of the year and

$$A(t) = \int_0^t [-f_1(\tau) - f_2(\tau) - m(\tau) + R(\tau)]d\tau. \tag{7}$$

The population is invariant over a year if $A(T) = 0$, where $T = 1$ year (or 365 days since t is in days). When $A(T) = 0$, the various removal processes just match recruitment into the fishery over the year.

The effect of each term included in the right side of equation (7) can be calculated by integrating the right side of equation (5) with respect to that term. For example, the cumulative number of oysters per area removed by fishing during the spring season ($O_{f_1}(T)$) is

$$O_{f_1}(T) = \int_0^T f_1(\tau)N(\tau)d\tau. \tag{8}$$

The structure of the various terms is required to proceed. First let recruitment R be an impulse or delta function at the beginning of the year, or

$$R(t) = R_o\, \delta(t - \epsilon), \tag{9}$$

where R_o is the total recruitment rate and ϵ is a small number to have recruitment occur shortly after $t = 0$. If the recruitment is expected to double the number of oysters at the beginning of the year, then $R_o = \log_e 2$. This impulsive form resolves the issue of dependency of recruitment into the fishery on N.

Each of the other terms is modeled with a box-like structure so that the process turns on and off at certain times. That is, each term turns on once and then turns off (Fig. 2). This formulation allows more than one process to be on at a time, so the model is completely general. Each term has the form

$$B(t : t_1, t_2) = \frac{1}{2}\left[\tanh\left(\frac{t - t_1}{a}\right) - \tanh\left(\frac{t - t_2}{a}\right)\right]. \tag{10}$$

The 'box' function described by equation (10) has the value one-half at the times t_1 and t_2, or $B(t_1) = B(t_2) = 0.5$ (Figure 2). The parameter a, with units of days, controls how fast the process turns on or off. So, for small a (at $a \ll 1$), $B = 0$ before $t = t_1$, B = 1 for $t_1 < t < t_2$ and $B = 0$ after $t = t_2$ (Figure 2). For a regulated behavior, such as fishing, where seasons open and close over the course of a single day, a small a permits the process of fishing mortality to rapidly switch on and off as required ($a = 0.5$ in Fig. 2). For disease mortality that waxes and wanes over a longer period of time, a larger a permits the rate to increase and decrease slowly over a period of weeks ($a = 10$ in Fig. 2).

The time integral of the box function is required to get the solution; that is, to get $A(t)$, so

$$\begin{aligned} BI(t; t_1, t_2) &= \int_0^t B(\tau)d\tau \\ &= \frac{a}{2}\left[\log\cosh\left(\frac{t - t_1}{a}\right) - \log\cosh\left(\frac{t_1}{a}\right)\right. \\ &\quad \left. - \log\cosh\left(\frac{t - t_2}{a}\right) + \log\cosh\left(\frac{t_2}{a}\right)\right]. \end{aligned} \tag{11}$$

Although it is not clear from this formula, $BI(t; t_1, t_2)$ $(t \gg t_2) = t_2 - t_1$. That is, the integral of $B(t; t_1, t_2)$ beyond the end of the 'box' defined by t_1 and t_2 is just the time the process is switched on, $t_2 - t_1$ (in days).

The various sources of mortality are represented as

$$(f_1, f_2, m) = (f_{10}, f_{20}, m_0)B(t; t_1, t_2) \tag{12}$$

where t_1 and t_2 are different for each process. So each of the fishing and mortality processes are controlled by a constant amplitude times the 'box' function which has start and stop parameters. Furthermore, the steady solution is constructed in parts as each of the processes can be integrated in time as

$$\int_0^t (f_1, f_2, m)d\tau = (f_{10}, f_{20}, m_0)BI(t; t_1, t_2). \tag{13}$$

The recruitment function used to calculate total recruitment, $\hat{R}_o$, is simple to integrate because of the pulse-like behavior:

$$\int_0^t R(\tau)d\tau = \int_0^t R_o\, \delta(\tau - \epsilon)d\tau = \hat{R}_o \tag{14}$$

as long as $t > \epsilon$. But this is always true after the first day.

Further development of the model requires establishment of a goal for the management program. One option, given the uncertainties in year-to-year variations in natural mortality that preclude evaluation of the stock abundance relative to a known value such as carrying capacity K, is to adopt the goal that market-size abundance should not decline over the year due to activities of the fishery. Stated mathematically:

$$N_{t=0} = N_{t=365}. \tag{15}$$

The condition expressed by equation (15) necessitates that the rate processes in equation (7) sum to zero [$A(T) = 0$] or

$$\hat{R}_o - f_{10}BI(T; t_1, t_2) - f_{20}BI(T; t_3, t_4) - m_0BI(T; t_5, t_6) = 0, \tag{16}$$

where t_1, t_2, t_3, t_4, t_5, and t_6 are the transition times for fishing and natural mortality. The form of the 'box' function means that each

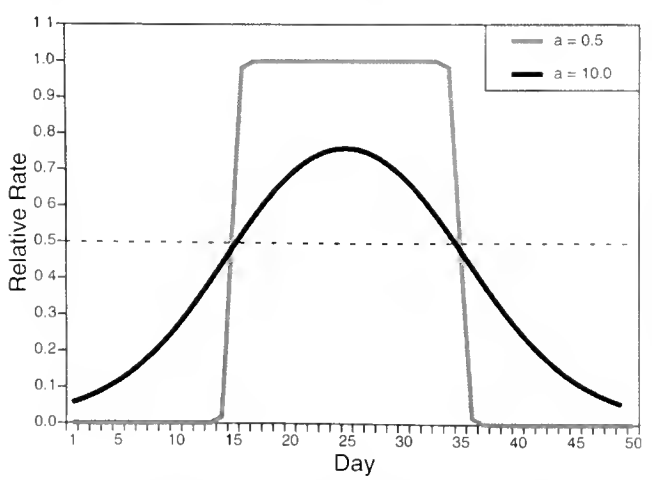

Figure 2. Examples of the time-dependent rate functions used for mortality rates (equation [10]). Grey line, the time-dependent fishing mortality rate, $a = 0.5$. Black line, the time-dependent *Perkinsus marinus* mortality rate, $a = 10$.

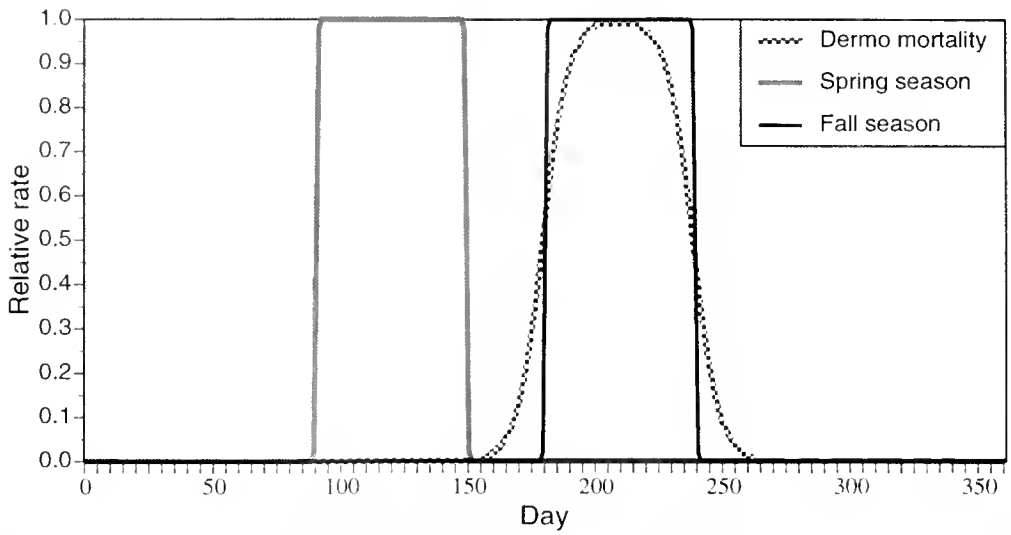

Figure 3. An example time series of mortality rates showing the two independent fishing seasons and the time of highest *Perkinsus marinus* induced mortality.

term in equation (16) reduces to the time span that each process is on times a constant amplitude. An example time series is shown in Figure 3. So, equation (16) becomes

$$\hat{R}_o - f_{10}(t_2 - t_1) - f_{20}(t_4 - t_3) - m_0(t_6 - t_5) = 0. \quad (17)$$

A little rearrangement gives

$$(t_2 - t_1)f_{10} + (t_4 - t_3)f_{20} = \hat{R}_o - (t_6 - t_5)m_0 \quad (18)$$

Equation (18) gives a relationship between the two fishery rates compared to recruitment and natural mortality.

The rest of the calculation is determined by the choice of the relationship between the two fishing mortalities. For example, suppose that the per day fishing rates for spring and fall are the same. This simply says that the daily effort and catch per unit effort are constant. In the case of the Delaware Bay oyster fishery, daily effort and CPUE (measured in terms of bushels d^{-1}) are very similar in the fall and spring and CPUE varies little between years because changes in stock abundance do not substantially influence CPUE (when measured in terms of bushels d^{-1}). (Banta et al., in press). Thus, $f_{20} \sim f_{10}$ and the fishing rates are easily estimated by:

$$f_{10} = \frac{\hat{R}_o - (t_6 - t_5)m_0}{t_2 - t_1 + t_4 - t_3}. \quad (19)$$

In some cases, such as after an epizootic, maintaining a stable population may not be desirable. In this case, the balance between recruitment and mortality established by equations (6) and (15–18) must be altered. Equation (6) becomes

$$N(t) = \frac{N_o}{\eta} e^{A(t)}, \quad (20)$$

where η is the desired ratio between the abundance of market-size oysters at the beginning of the year ($N_{t=0}$) and at year's end ($N_{t=365}$): $N_{t=0}/N_{t=365} = \eta$. Equation (20) can be rearranged to

$$N(t) = N_o e^{[A(t) - \log_e(\eta)]}. \quad (21)$$

Equation (17) then becomes

$$\hat{R}_o - f_{10}(t_2 - t_1) - f_{20}(t_4 - t_3) - m_0(t_6 - t_5) = \log_e(\eta). \quad (22)$$

Once again, letting $f_{10} = f_{20}$ provides a relationship analogous to equation (19).

$$f_{10} = \frac{\hat{R} - (t_6 - t_5)m_0 - \log_e(\eta)}{t_2 - t_1 + t_4 - t_3}. \quad (23)$$

TABLE 1.

The total number of bushels of market-size and submarket-size oysters on the Delaware Bay oyster beds in late-October, 1999, listed in bushels based on an estimated 348 market-size oysters per bushel and 499 submarket-size oysters per bushel.

Seed Bed	Market-size Oysters	Submarket-size Oysters
Transplant Group		
Low-mortality		
Arnolds	3,182	11,646
Round Island	1,954	3,847
Upper Arnolds	3,412	12,410
Medium-mortality		
Upper Middle	0	0
Middle	19,150	30,535
High-mortality		
Beadons	899	4,017
Nantuxent Point	3,047	8,691
Direct-market Group		
Medium-mortality		
Cohansey	72,936	127,380
Sea Breeze	11,226	6,246
Ship John	39,293	45,325
Shell Rock	8,258	21,620
High-mortality		
Bennes	7,470	16,632
Bennies Sand	951	1,931
Egg Island	462	618
Hawk's Nest	9,447	16,774
Hog Shoal	1,681	1,857
New Beds	4,840	7,626
Strawberry	1,279	2,651
Vexton	1,815	1,857

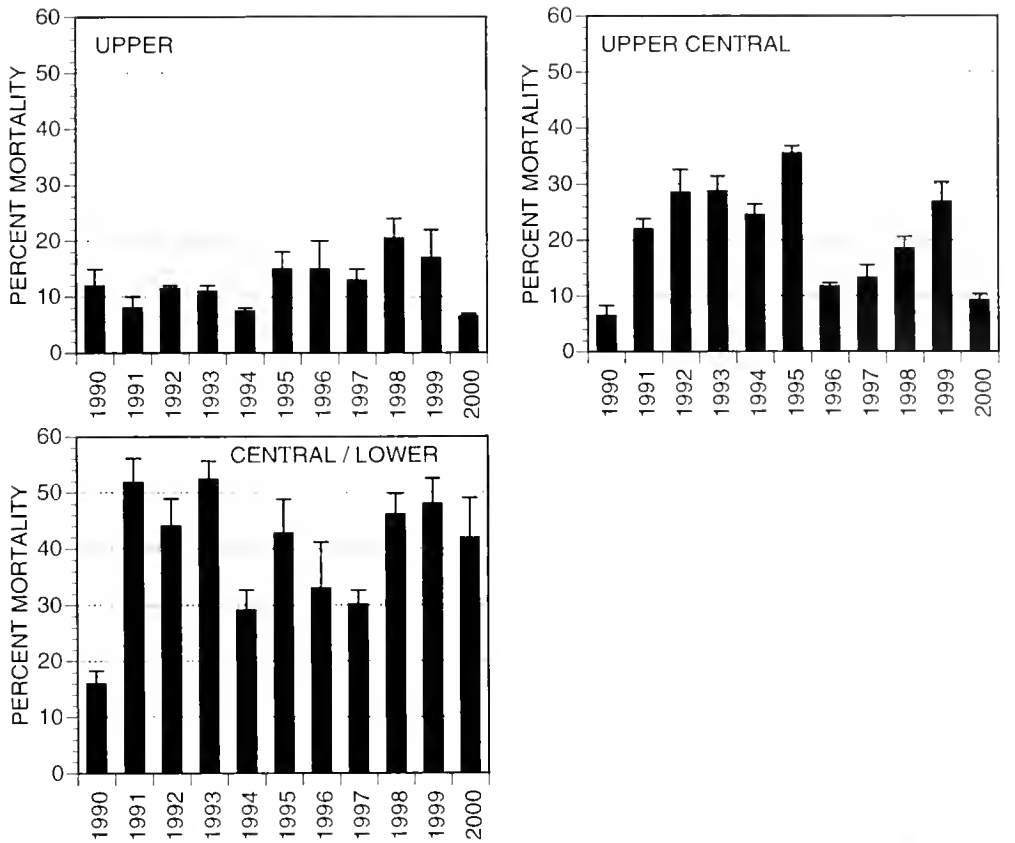

Figure 4. Yearly estimated natural mortality as a percent of total market-size abundance for the three bed groupings defined in Table 1, for the decade of the 1990s. Dermo became a significant source of mortality in 1990; as a consequence, mortality for years prior to 1990 is not included in the time series.

Data

The data come from the Delaware Bay oyster stock assessment program that has produced a yearly autumnal survey of the seed beds since the early 1950s. A stratified random sampling method is used for the survey. Each bed (Fig. 1) is divided into a series of contiguous 25-acre grids that fall into one of three strata. The strata consist of "test" areas, typifying the highest quality areas of the bed that sampling over the course of many years has shown to have a high percentage of living oysters 75% or more of the time, "high quality" areas in which oysters were abundant 25–75% of the time, and "low quality" areas in which oysters were abundant less than 25% of the time. The survey consists of about 100 samples covering the primary and most of the minor oyster beds. Each sample represents a composite of 3 one-third bushels from three randomly-directed one-minute tows within each sampled grid. The survey instrument is a standard 1.27-m commercial dredge on a typical dredge boat, the *F/V Howard W. Sockwell* in this case. Oyster abundance is quantified in terms of numbers per m^2 by correcting the raw counts obtained from the three measured (by DGPS navigation) tows by a measure of dredge efficiency (Powell et al. submitted). Data from the 2000 survey are used in model simulations (Table 1).

The seed beds were divided into three groups based on the level of natural mortality normally experienced during the 1990s (HSRL 2001). These groups will be referred to as the low-mortality, medium-mortality, and high-mortality groups (Table 1, Fig. 4). The bed groupings follow the salinity gradient, as anticipated from the importance of Dermo disease in the mortality pattern, such that the lowest natural mortality rates are on the upbay, lowest-salinity beds and the highest natural mortality rates are on the downbay, highest-salinity beds. The time series of natural mortality since 1990, when Dermo began to become an important source of mortality in Delaware Bay, is characterized by two Dermo epizootics, an extended epizootic in 1991–1995 and a shorter epizootic in 1998–1999. Percentiles of natural mortality rate were calculated from the rank-order of yearly mortalities for 1990–2000 obtained from this time series.

The oyster seed beds were also divided into two groups based on the estimated size of the smallest individual that could recruit to the fishery during one year. These sizes were 63.5 mm for all high-mortality beds (listed in Table 1) with the exception of Shell Rock and 70.0 mm for all other beds (HSRL, 2001). Finally, some beds, as shown in Table 1, are normally used in a transplant program; others are used for direct-marketing. The distinction is unimportant for this treatment, but is retained to maintain consistency with referenced sources (e.g., HSRL, 2001).

RESULTS

Compensation Between Fishing and Natural Mortality

The importance of compensatory mortality was examined using a Dermo mortality rate characteristic of epizootic conditions on the high-mortality beds (Figure 4): 0.5 yr^{-1}. Simulations were run for cases with two fishing seasons, the first opening and closing before

P. marinus reaches levels capable of generating mortality in the population and the second varying in its timing with respect to the time period of highest mortality from disease (Table 2). Simulations were run under the provision of no net change in market-size abundance over the year. Values used for the simulations were the sums of the estimated abundances of market-size and submarket-size oysters for the high-mortality direct-market beds (Table 1). In past years, these beds have supported the vast bulk of industry effort in Delaware Bay (Banta et al., in press).

Appropriate timing of the fishing season with respect to the timing of disease mortality can more than double a seasonal allocation to the fishery under the constant abundance scenario (Table 2). In the twenty simulations provided in Table 2, the highest yearly fishing allocation is 43,261 bushels. The lowest allocation is 33,969 bushels, about 20% less than the highest value. Differences in allocation often reach 50% within an individual fishing season. For example, in the first set of simulations in Table 2, the second fishing season was translated in time with respect to the timing of Dermo-induced mortality. Setting the second fishing season prior to the time of greatest disease mortality provides an allocation of 18,575 bushels. Setting the season after disease mortality ceases provides an allocation of 9,361 bushels. In each of these cases, population abundance at the end of the year is the same. Not surprisingly, varying the timing of disease mortality relative to a designated fishing season, examples of which are shown in the second group of simulations in Table 2, has a similarly profound effect on the yearly allocation.

In Delaware Bay, the oyster season normally opens in early April. Many times, the season has been split into two parts, one from April to June and one from September to November. An alternative is one long season beginning in April and extending into the summer or early autumn. To the extent that this season terminates prior to the late summer rise in disease mortality, the yearly allocation will increase over the alternative of a fall season. However, as the summer season begins to overlap the period of highest disease mortality, the yearly allocation will decrease as some oysters, otherwise harvestable, succumb to disease. An extreme example in which the fishing season is varied such that more and more of the season coincides with the time of greatest disease mortality is shown in the third group of simulations in Table 2. In this case, disease mortality occurs for a short, but intense, time in late summer. Total lost allocation reaches about 16% when the fishing season extends into and after the season of most intense natural mortality.

Historically, the fall harvest has obtained a higher market price. Hence economic issues encourage harvesting after Dermo disease has waned. However, this strategy minimizes the yearly allocation. Simulations in Table 2 suggest that the per-bushel dock-side price must at least double to compensate for the reduction in yearly allocation in epizootic years when the rate of natural mortality is high. A doubling of price is, in fact, rarely achieved, suggesting that the attraction of the higher market price in the fall is more often than not false economics in the oyster industry in these years. Harvest and, probably, dock-side value is maximized by concentrating the fishery, to the extent possible, prior to the time that disease mortality increases in late summer.

TABLE 2.

The estimated yearly allocation for spring and autumn fishing seasons during a year when 50% of the market-size oysters succumbed to Dermo disease. Day 1 was set at January 1. Data are presented in bushels. One bushel ~37 L and contains approximately 348 market-size oysters.

Fishing Mortality				Year Days		Year Days		
Total (In Bushels)	Season 1 (In Bushels)	Season 2 (In Bushels)	Dermo Mortality (In Bushels)	90	150	150	240	330
43,261	24,686	18,575	28,797					
41,872	24,686	17,186	30,185					
38,307	24,686	13,621	33,750					
35,080	24,686	10,394	36,977					
34,048	24,686	9,361	38,010					
33,961	24,600	9,361	38,096					
35,080	24,686	10,394	36,977					
38,307	24,686	13,621	33,750					
41,872	24,686	17,186	30,185					
43,261	24,686	18,574	28,795					
43,261	24,686	18,575	28,797					
42,176	20,287	21,888	29,881					
40,044	17,214	22,829	32,013					
37,927	14,948	22,978	34,130					
36,228	13,208	23,020	35,829					
42,560	24,685	17,874	29,497					
40,526	24,686	15,840	31,531					
38,039	24,686	13,353	34,018					
35,932	24,686	11,246	36,125					
34,512	24,686	9,826	37,545					

Solid lines show the timing of the two fishing seasons. Dashed line shows the timing of Dermo mortality.

Variation in Disease Mortality Rate

Timing of disease mortality varies from year to year and is often difficult to measure in a timely fashion for near-real-time re-evaluations of management decisions. Varying the duration of disease-mortality from 60 to 120 days, as shown in simulation sets 1 and 4 in Table 2, has only a minor influence on cumulative yearly allocation, however, as long as the yearly natural mortality rate does not change. The daily rate is lower in the case of the longer time course, but the cumulative mortality integrated over the year is about the same.

Small variations in the natural mortality rate typically have profound consequences in fisheries models (Clark 1999), and this model is no exception. The yearly loss to Dermo disease is determined by a number of factors, including temperature, salinity, food supply, and the history of disease within the populations, that determine late summer and fall infection intensity (Hofmann et al. 1995, Powell et al. 1996, Cook et al. 1998, Soniat et al. 1998). Variation in the rate of Dermo-induced mortality significantly affects the yearly allocation estimated by the model (simulation set 1, Table 3). For example, the yearly allocation more than doubles with a decrease in natural mortality rate from 0.5 yr^{-1} to 0.3 yr^{-1}. This range of natural mortality rates is routinely observed in Delaware Bay (Fig. 4). Not surprisingly, the significance of compensatory mortality in determining the preferred timing of the second fishing season declines at low natural mortality rates (simulations sets 1, 2, and 3, Table 3).

The precautionary approach to fisheries management (FAO, 1995, Restrepo et al. 1998; see also Francis & Shotton 1997) would dictate the choice of a relatively high natural mortality rate in forecasting yearly allocations, perhaps 0.5 yr^{-1} for Delaware Bay. Variation in the timing of the fall fishing season significantly affects the yearly allocation, a difference of about 8,000 bushels in the simulations presented in Table 3. In contrast, even a small drop in natural mortality rate, from 0.5 yr^{-1} to 0.4 yr^{-1}, increases the allocation by about 10,000 bushels. An increase to 0.6 yr^{-1} essentially eliminates the yearly harvest. Thus, the model, and the population, are very sensitive to year-to-year variations in natural mortality rate. Precautionary management designed to minimize the likelihood of an underestimate in natural mortality rate is one consequence of this sensitivity. Increased predictability in the time course of Dermo disease, permitting a less conservative choice of natural mortality rate, would substantially increase the yearly allocation.

Influence of Spatial Variations in Natural Mortality

Spatial changes in the rate of natural mortality, as exist along the salinity gradient, may exert a significant influence on the estimated yearly allocation. Is this variation important in the practical context of management decision-making? The Delaware Bay oyster beds were partitioned into three groups according to the long-term average rate of natural mortality, the principal source of which is *P. marinus* infection. Yearly allocations to the oyster industry were evaluated for each of the three bed groups under a

TABLE 3.

The estimated yearly allocation for spring and autumn fishing seasons as a function of natural mortality rate for three different timings for the autumn fishing season. Day 1 was set at January 1. Data are presented in bushels. One bushel ~37 L and contains approximately 348 market-size oysters.

Dermo Mortality Rate (yr^{-1})	Fishing Mortality			Dermo Mortality (In Bushels)	Year Days		Year Days		
	Total (In Bushels)	Season 1 (In Bushels)	Season 2 (In Bushels)		90	150	150	240	330
0.01	71,511	39,534	31,976	546					
0.10	66,314	37,154	29,159	5,734					
0.20	59,854	34,084	25,769	12,204					
0.30	52,469	30,424	22,044	19,589					
0.40	43,845	25,949	17,895	28,212					
0.50	33,480	20,287	13,192	38,577					
0.60	20,496	12,771	7,724	51,561					
0.70	3,158	2,037	1,121	68,899					
0.01	71,710	46,705	25,005	347					
0.10	68,275	44,065	24,209	3,783					
0.20	63,662	40,624	23,037	8,395					
0.30	57,901	36,470	21,430	14,156					
0.40	50,482	31,317	19,165	21,575					
0.50	40,526	24,686	15,840	31,531					
0.60	26,371	15,703	10,668	45,686					
0.70	4,395	2,540	1,854	67,662					
0.01	71,754	46,704	25,049	304					
0.10	68,718	44,065	24,653	3,339					
0.20	64,567	40,624	23,943	7,490					
0.30	59,266	36,470	22,796	12,791					
0.40	52,260	31,316	20,943	19,797					
0.50	42,560	24,685	17,874	29,497					
0.60	28,227	15,703	12,523	43,830					
0.70	4,832	2,540	2,292	67,225					

Solid lines show the timing of the two fishing seasons. Dashed line shows the timing of Dermo mortality.

range of natural mortality rates that covered the range observed in these bed groups during the 1990s. For ease of comparison, the same oyster abundances were used for each bed group. This abundance was the 2000 abundance for the high-natural-mortality group (Table 1).

Not surprisingly, a much higher allocation was available for the low-mortality beds than for the high-mortality beds in these simulations (based on equivalent abundances between groups) (Figure 5). As before, the yearly allocation declined as natural mortality increased for each bed group. The range of allocations across the observed range of yearly mortality rates, a range that is assumed to document the range of anticipated mortality rates for a coming year, was much larger for the high-mortality group than for the other two groups.

In practice, the fishery operates principally on the high-mortality beds, hence the use of these abundance values in the simulations shown in Figure 5. A factor of two exists between the highest and lowest allocations for these beds, depending on the natural mortality rate that might occur in any given year. Obviously, errors in the prediction of natural mortality rate are most grave for this group. In fact, if stable market-size abundance is the desired outcome, taking the median rate of natural mortality for the decade of the 1990s for the low- and medium-mortality groups as the value for the coming year would introduce no more than about a 12% error in achieving this goal on these beds, even if natural mortality rate approached either the highest or lowest recorded values. In contrast, the error introduced for the high-mortality group would exceed 30%. Thus, increased levels of natural mortality increase the need for a precautionary approach to stock management given the limited ability at present to predict the course of Dermo disease (Powell et al. 1996, Soniat & Kortright 1998). For the high mortality group, use of the 64*th* or 75*th* percentile would seem appropriately conservative.

Influence of Variations in Growth Rate or Pre-recruit Abundance

Variation in the number of recruits into the fishery in a given year is also important. The premise of the model is to manage the fishery under the proviso of no net change in market-size abundance. Accordingly, the allocation is a function of the availability of new recruits and the natural mortality rate. The number of new recruits is controlled not only by settlement and recruitment into the population and subsequent survival of juveniles, but also by the yearly growth rate that establishes the smallest-sized oyster anticipated to recruit to the fishery during the season. Because the number of oysters typically declines in the larger size classes, slower growth can have a dramatic effect on yearly allocation by reducing the number of new recruits.

The simulations shown in Figure 5 were based on the assumption that oysters 63.5–76 mm in size recruit to the fishery in a given year (market size, ≥76 mm). This is a growth rate typical of the higher-salinity oyster beds in Delaware Bay. Growth rate slows upbay. The equivalent simulations are shown for the case where oysters 70–76 mm are assumed to recruit to the fishery during the season in Figure 6. The difference in allocation is based on the observed number of oysters in these two size-classes on the high-mortality beds in 2000. About 47% of the oysters in the 63.5–76-mm size-class were ≥70 mm in size.

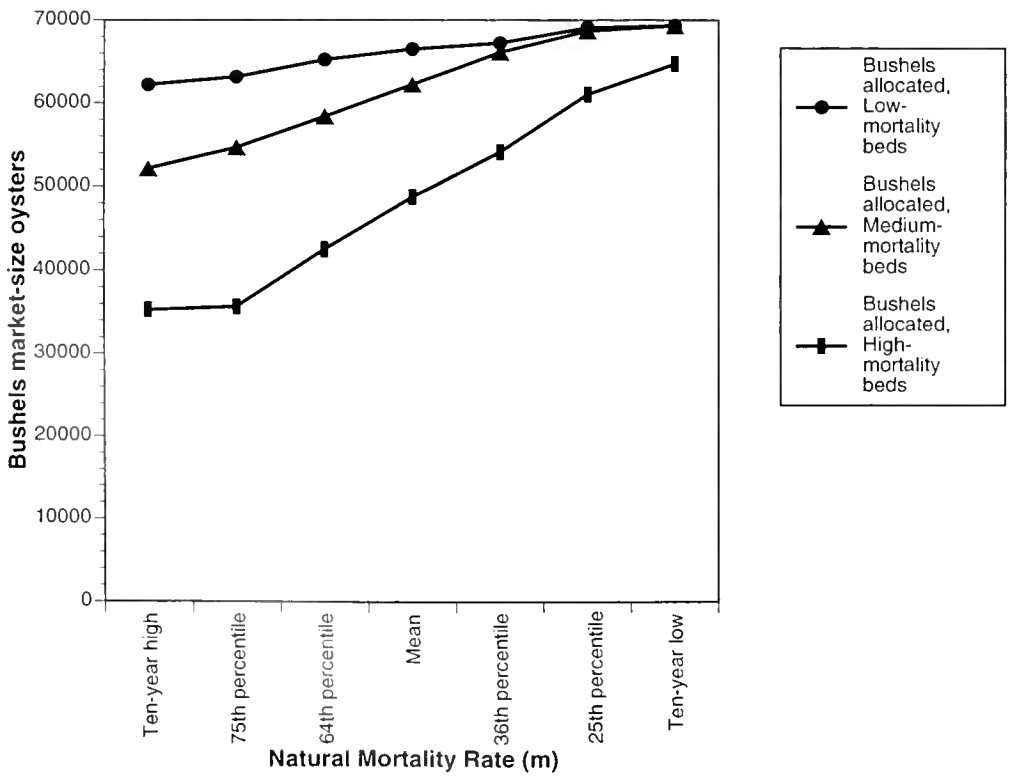

Figure 5. Yearly allocation (in bushels) for various rates of natural mortality (principally caused by *Perkinsus marinus*). Simulations were run for three groups of beds characterized by low, medium and high rates of natural mortality and for the range of natural mortality rates observed during the decade of the 1990s for these beds (Figure 4). In each case, the fishing season was initiated on day 90 and ceased on day 288 (the 2001 fishing season for New Jersey). *Perkinsus marinus*-mortality occurred between days 180 and 300 at the yearly rate shown in Figure 4. Oysters 63.5–76 mm were assumed to recruit to the fishery.

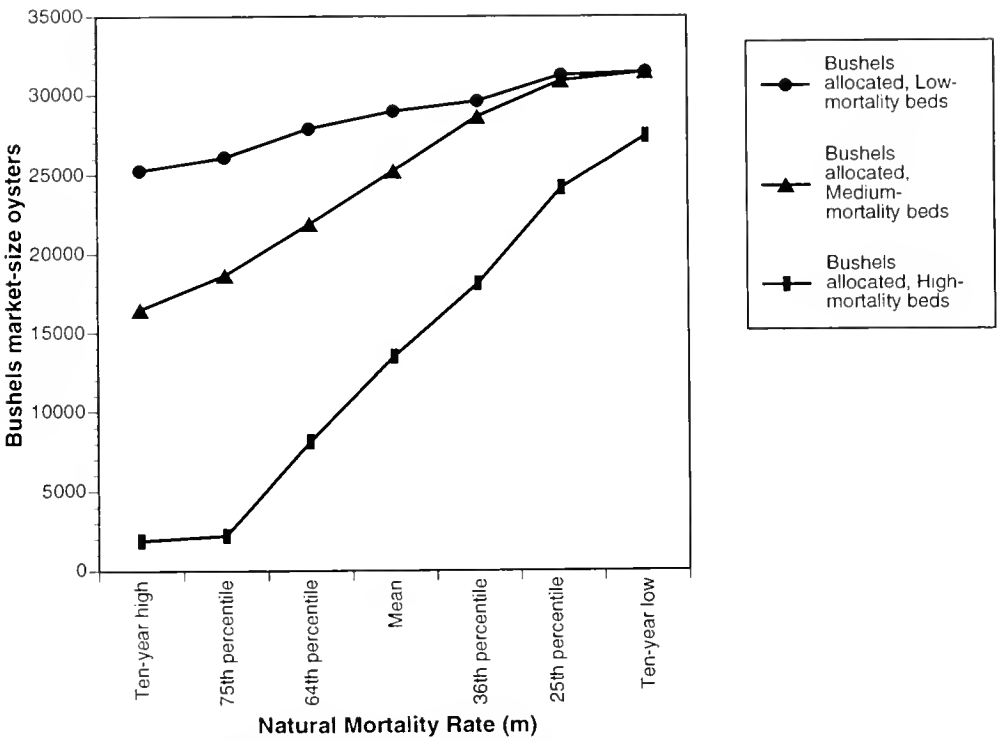

Figure 6. Yearly allocation (in bushels) for various rates of natural mortality (principally caused by *Perkinsus marinus*). Simulations were run for three groups of beds characterized by low, medium and high rates of natural mortality and for the range of natural mortality rates observed during the decade of the 1990s for these beds (Figure 4). In each case, the fishing season was initiated on day 90 and ceased on day 288 (the 2001 fishing season for New Jersey). *Perkinsus marinus*-mortality occurred between days 180 and 300 at the yearly rate shown in Figure 4. Oysters 70–76 mm were assumed to recruit to the fishery.

A reduction in the number of recruits has two significant effects. First, the total yearly allocation is significantly lower under the "slow-growth" assumption (compare Figs. 5 and 6). In addition, the range in allocation is increased when evaluated across the range of natural mortality rates observed during the decade of the 1990s. For the high-mortality beds that are most sensitive to this effect, the allocation estimated from the median rate of natural mortality for the decade of the 1990s is now a factor of 2 different from the extreme values rather than the ~30% difference observed with the higher growth rate in Figure 5. As growth slows, the importance of an accurate estimate of anticipated natural mortality rate increases and, as a practical matter, the more precautionary must be the management evaluation prior to the season.

Post-Epizootic Population Recovery

Natural mortality rates during Dermo epizootics may exceed 0.7 yr^{-1} (Mackin 1959, Andrews 1988, Powell et al. 1996). Consequently, oyster population abundance typically declines significantly during epizootic years. Management of the oyster resource might include rebuilding stocks after an epizootic. Management goals might include a return to the long-term average population level or some higher level (e.g., 75*th* percentile).

In Delaware Bay, 2000 end-of-year weighted-mean oyster abundance (calculated as # $\geq$ 76 mm + 0.5 × #63.5 – 76 mm) was near the decadal average weighted-mean oyster abundance (1989–2000) and about 67% of the decadal 75*th* percentile weighted abundance (Fig. 7). Thus, a one-year return to the 75*th* percentile weighted abundance would require an end-of-year ratio of $N_{2001}/N_{2000} = 1.50$.

Figure 8 shows the relationship between fishery allocation, natural mortality rate, and a desired degree of increase (or decrease) in market-size abundance at year's end. The 2000 allocation for Delaware Bay was 40,000 bushels for the entire bay (all beds), a low level required by the ongoing Dermo epizootic at that time. Approximately the same allocation could be achieved in 2001 just on the high-mortality beds, under the management goal of no net change in oyster abundance (Fig. 8, upright triangles). This option exists because a significant increase in juvenile oyster abundance occurred in 2000 that dramatically increased the number of new recruits available to the fishery in 2001 (Fig. 9). However, because the number of bushels available for harvest on the medium-mortality beds upbay was about 60,000 bushels (HSRL 2001) an opportunity would exist to rebuild population abundance on the high-mortality beds and still increase the yearly allocation. Figure 8 shows that rebuilding to half of the 75*th* percentile abundance goal ($N_{2001}/N_{2000} = 1.24$) can be accomplished even at a relatively high natural mortality rate, if the allocation on the high-mortality beds is reduced to about 20,000 bushels. Reaching the 75*th* percentile weighted abundance goal is achievable if the yearly allocation on these beds is limited to about 8,000 bushels (Fig. 8). Even higher abundances could be achieved; however, only if the natural mortality rate is at or below the long-term mean. Thus, recovery from the 1998–1999 epizootic with a return to above average oyster abundances could be accomplished in one year even under the precautionary management assumption that natural mortality rates will approach the 75*th* percentile of the decadal time series.

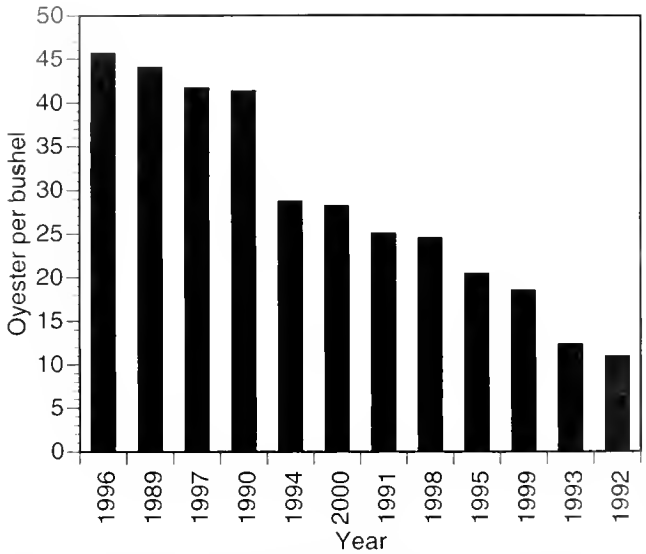

Figure 7. Rank-order of oyster weighted-mean abundance (calculated as # ≥76 mm + 0.5 × # 63.5–76 mm) for the high-mortality Delaware Bay oyster beds for 1989–2000.

DISCUSSION

Setting the yearly allocation for a fished stock is always an uncertain endeavor. Choice of the natural mortality rate is critical in most fisheries models. Populations suffering significant mortality from disease, particularly those where year-to-year changes in mortality rate cover a wide range, require particularly careful management. Unfortunately, routinely-used fisheries models do not consider mortality from disease.

Compensatory mortality is frequently observed in populations where disease mortality is significant. Some animals dying in other ways would otherwise have died from disease. In a commercial species, fishing mortality is an important additional source of mortality and compensatory processes should be important. Thus, a fisheries model developed to evaluate a diseased population must be capable of resolving compensatory mortality originating from the timing of the various sources of mortality during the year.

The model presented here permits the evaluation of compensatory processes, thereby giving guidance to appropriate timing of fishing seasons for maximizing yield. In some cases, the economic attraction of a fall harvest may be offset by the decreased yield available to the fishery. An earlier season permits a higher harvest because some of the animals taken by the fishery would subsequently be lost to the population through disease.

Although a number of shellfish models exist (Kobayashi et al. 1997, Powell et al. 1997, Dowd 1997), few fisheries models tailored specifically to shellfish exist (e.g., NEFSC, 2000a, 2000b). Fisheries models require a forward prediction of abundance under a chosen natural mortality rate m. However, small changes in mortality rate effect large changes in predicted outcomes. The present model is no exception. Outcomes are strongly influenced by the chosen yearly mortality rate. Accordingly, the accuracy of

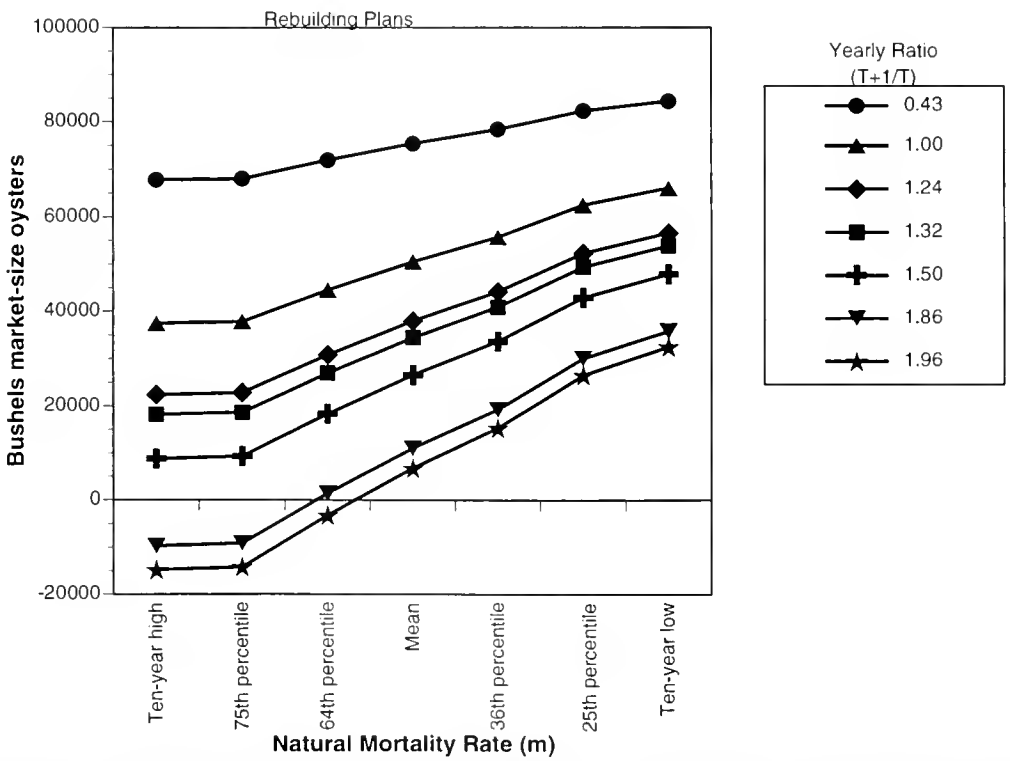

Figure 8. Yearly allocation (in bushels) for various rates of natural mortality (principally caused by *Perkinsus marinus*). Simulations were run for the high-mortality beds over a range of natural mortality rates observed during the decade of the 1990s (Figure 4). Each simulation was run to produce a desired change in market-size oyster abundance over the year, calculated as $N(T+1)/N(T)$. In each case, the fishing season was initiated on day 90 and ceased on day 288 (the 2001 fishing season for New Jersey). *Perkinsus marinus*-mortality occurred between days 180 and 300 at the yearly rate shown in Figure 4. Oysters 63.5–76 mm were assumed to recruit to the fishery. Negative numbers indicate cases where an insufficient number of new recruits was available to meet the demands of the rebuilding program.

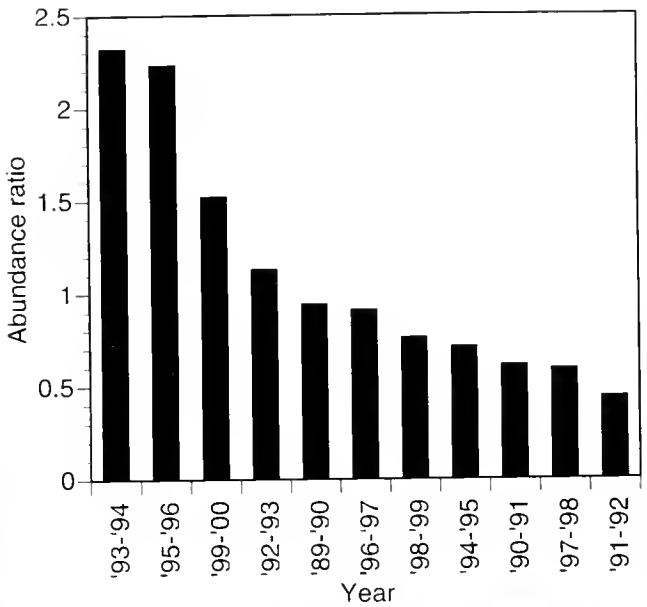

Figure 9. Rank-order of the year-to-year change in oyster weighted-mean abundance (calculated as # ≥76 mm + 0.5 × # 63.5–76 mm) for the high-mortality Delaware Bay oyster beds for 1989–2000.

forward prediction is controlled, in part, by the accuracy at which the disease process can be predicted in oyster populations.

Besides disease, the other model parameter that most affects the outcome of simulations is the abundance of submarket-size oysters that can be expected to recruit to the fishery in the simulated year. A small drop in growth rate can dramatically alter the estimate of the yearly allocation because market-size is on the tail end of the size-frequency distribution. Thus, the definition of submarket size is critical. In addition, the abundance of submarket-size individuals establishes the resilience of the population to transient increases in disease-induced mortality and to errors in management that lead to overfishing. As the number of submarketsize oysters declines, sustainability of the population becomes less certain and the accuracy of model simulations becomes more critical. In essence, as the ratio of submarket to market-size abundance approaches 1.0, reliance on more conservative management approaches should increase.

Although the maximum life span of *Crassostrea virginica* is at least 15 yr (Lavoie & Bryan 1981), the average life span in most East and Gulf coast bays probably does not exceed four due to fishing and disease. Thus, population stability is strongly determined by the number of recruits available to replace the deaths that decimate the market-size population each year. In Delaware Bay in 2000, the number of recruits was insufficient to permit rapid recovery from the most recent *P. marinus* epizootic without conservative management measures because the epizootic resulted in lower survival of the submarket-size class, a frequent characteristic of epizootic conditions (Powell et al. 1996), and because spat settlement rates were low prior to this time interval (HSRL 2001). In 2001, conditions changed. An increase in recruits to the fishery provided an opportunity to rebuild population abundances to levels above the decadal mean, while still permitting an increase in allocation over 2000. This modeling exercise points to the critical need to understand population dynamics and survival of size classes below market size that many times are not the targets of investigatory activities (Hofmann et al. 1995).

The objective of management of the oyster fishery should be the maximization of yield under conditions that stabilize population abundance at some preferred level. What that preferred level is cannot be ascertained by the model described here. However, once that preferred level is chosen, the model presented here permits the evaluation of management scenarios targeting this objective. The model requires a quantitative estimate of abundance by size class, some knowledge about growth rates to establish the size range recruiting into the fishery in any given year, and a decision about the natural mortality rate anticipated. The latter is of considerable consequence and, so, probably should be chosen conservatively, perhaps a value at or above the long-term average. Once these data are available, the model permits investigation of scenarios that include a range of allocations, the timing of fishing seasons, and the variation of fishing efforts within seasons in order to establish a preferred harvest level for the oyster fishery. The model also supports investigation of recovery scenarios after an epizootic and the evaluation of population resiliency to future epizootics or overestimates of fishery yield. The model is sufficiently general that it likely can be applied to any commercial shellfish species, providing that the stock assessment program provides survey data adequate for the modeling exercise.

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A DNA PROBE FOR TRANSCRIPTION ANALYSIS OF THE PROTEOLYTIC ENZYME CATHEPSIN B IN THE PACIFIC OYSTER, *CRASSOSTREA GIGAS* (THUNBERG, 1793)

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ABSTRACT Molecular techniques have been employed to develop a probe for the proteolytic enzyme cathepsin B in the Pacific oyster, *C. gigas*. Degenerate primers were used to amplify a 450 base pair (bp) fragment of the cathepsin B gene. Deduced amino acid sequence indicates 60, 59, and 59% identity with cathepsin B from human, rat and cow, respectively. Expression of cathepsin B was detected, by RT-PCR, in both larval and adult tissues. Northern blot analysis demonstrated expression of a single transcript of approximately 2.4 kb.

KEY WORDS: proteolytic enzymes, gene expression, cathepsin B, *Crassostrea gigas*

INTRODUCTION

The continuous breakdown, replacement and/or renewal of proteins, known as protein turnover, is an essential component of metabolism, enabling development, adaptation, repair and regulation (Hawkins 1991, Ciechanover & Schwartz 1994). Nevertheless, the intensity of protein turnover varies greatly according to genetic and environmental influences, with significant consequences for growth and survival among animals generally (Hawkins & Day 1999). Comparisons between individual shellfish have shown that whole-body specific activity of the key proteolytic enzyme cathepsin B correlated with the intensity of whole-body protein turnover, and was associated with both higher energy expenditure and slower growth rate (Hawkins & Day 1996). Cathepsin B is a proteolytic enzyme belonging to the papain cysteine protease superfamily (Hasnain et al. 1993), and exhibits both endo- and exopeptidase activities (Koga et al. 1991). Mammalian cathepsins have been extensively studied, due to the correlation between cathepsin activity and cancers and brain disorders, such as Alzheimer's disease and multiple sclerosis (Bernstein 1994). In comparison, cathepsin activity in invertebrates has received comparatively little attention (Zeef & Dennison 1988).

Here, to enable future work elucidating mechanisms that regulate cellular levels of cathepsin B, we describe the development of a molecular probe to monitor expression of the cathepsin B gene in the commercially important Pacific oyster *Crassostrea gigas* at different stages of development and in different adult tissues. During development, there are "critical windows" of increased protein turnover, when major structural changes occur as *C. gigas* individuals develop into different larval forms and then metamorphose from a free-swimming planktonic larva into a sessile calcified adult. Within five to 10 hours of fertilization, a non-feeding trochophore larva is formed, which within 24 to 48 hours develops into a planktotrophic, straight-hinge veliger larva (Dekshenieks et al. 1993). Between two and three weeks PF, the larva develops a foot and a pair of eyespots. This is the pediveliger form. Once the larva reaches ~300 μm, usually after approximately 25 days PF when grown at 23°C, metamorphosis occurs. This involves a major anatomical reorganization: the velum and foot are lost, the organ systems are rearranged and the gill and adductor muscle become enlarged (Galtsaff 1964). Following metamorphosis, *C. gigas* remain benthic and undergo no further re-organization of their body plan. Proteolysis continues to maintain normal functions, such as sanitation and breakdown of degraded proteins (Ciechanover & Schwartz 1994). However we do not expect levels to be as high as in the larvae, due to substantial changes in body plan and a weight specific decrease in metabolic rate. The only exception to the relatively low levels of protein turnover expected in adult tissues may be in the gonads immediately prior and post spawning, as high energy expenditure and protein turnover is necessary for spawning to take place (Peek & Gabbott 1990).

In summary, we have used molecular techniques to develop a probe to detect transcripts of the proteolytic enzyme cathepsin B gene in *C. gigas*. We have used the cathepsin B probe to quantify cathepsin B gene transcripts at times of expected high and low energy expenditure and protein turnover, which included key points in larval development and different tissues from adult oysters.

MATERIALS AND METHODS

DNA Extraction

Crassostrea gigas individuals from Whitstable oyster hatchery were acclimated for a minimum of seven days in a system of recirculated sea water at 15°C. Following this acclimation adductor muscles were dissected from individual oysters and ground under liquid nitrogen. Fifty milligrams of the ground tissue was transferred to 500 μl extraction buffer (50 mM Tris-HCl pH8, 100 mM NaCl, 50 mM EDTA, 1% SDS). Proteinase K (50 μg) was added and the solution incubated at 55°C for 1 hour. Phenol:chloroform extraction was performed on the solution and DNA precipitated by incubation with 0.1 volumes 3M NaOAc and 2.5 volumes 100% ethanol at −20°C overnight. The precipitated DNA was harvested by centrifugation, washed in 70% ethanol and dried in a vacuum desiccator. The dried pellet was re-suspended in 500 μl TE and incubated with 5 μl RNase (10 $mg.ml^{-1}$) at 37°C for 30 minutes and then with 10 μl proteinase K (10$mg.ml^{-1}$) at 55°C for 30 minutes. A second phenol:chloroform extraction and ethanol precipitation were then performed and the DNA harvested by centrifugation. The pellet was washed in 70% ethanol, dried by vacuum desiccation and re-suspended in 500 μl TE. The purity and quantity of DNA was measured spectrophotometrically and by agarose gel electrophoresis.

PCR-Amplification

Amplification of genomic DNA was performed using 100 ng extracted DNA template per reaction. Degenerate primers were

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designed from conserved regions of the cathepsin B gene, using the sequence entries in the EMBL database and software at the Human Genome Mapping Project Resource Centre (HGMP-RC), Cambridge UK (see Fig. 1).

Cath1f: 5′-gg(a/c/t) tg(c/t) (a/c/g)a(a/g/t) ggt (a/g)g(a/c) tat-3′; Cath1r: 5′-ga(a/g) ttg gc(a/c/g/t) a(c/t)(a/c) agc cag-3′

The initial cycling parameters included a denaturation step of 92°C (5 min), followed by 35 cycles of 48°C (1 min), 72°C (1 min), and 92°C (1 min). A final annealing phase at 48°C (2 min) was followed by an extension phase at 72°C (5 min) and storage at 4°C. Due to the limited quantities of initial PCR product, a second amplification was performed with identical primers using 5 μl of the initial PCR product as template and with the annealing temperature increased to 50°C and the number of cycles reduced to 30. Aliquots of the second amplification reaction were visualized by agarose gel electrophoresis (1.2%).

Sequencing

To verify that the correct gene had been amplified, the PCR product was cloned into the *Sma*I site of pBluescript SK⁻, to produce plasmid p*CG*cathI, and sequencing was performed following the dideoxy chain termination method (Sanger et al. 1977) and using the Sequenase II sequencing kit (Amersham).

Transcript Detection

Two methods were used to detect CathB transcripts, reverse transcription (RT)-PCR and northern blotting. There are merits and drawbacks to both techniques. During RT-PCR, RNA is reverse transcribed to make cDNA, which is then amplified by PCR using gene specific primers. RT-PCR is a very sensitive technique. However it is non-quantitative and can only be used to look for the presence/absence of any expressed gene. Also, transcript size cannot be determined using RT-PCR as only part of the transcript is amplified. Northern blotting involves the hybridization of a radiolabelled probe to size separated transcripts. This technique can be used to identify transcript size and for quantitation, but is less sensitive than RT-PCR.

RNA Extraction and RT-PCR

RNA was extracted from adults and from larvae at "critical windows" in development when protein turnover was expected to be high; at one day post-fertilization the larvae is developing from the trochophore into the veliger form and around settlement the larvae changes from a free-swimming larvae to a calcified adult. At both these stages energy expenditure and metamorphosis are high. Adult tissue, dissected from six individual oysters several days post spawning, and larvae, sampled at one day PF and around settlement metamorphosis, were obtained from the Whitstable oyster hatchery. The oysters were prepared for RNA extraction by grinding the adult tissue under liquid nitrogen and homogenizing the larvae in lysis buffer. RNA was then extracted using the SV total RNA isolation system (Promega). The quantity and purity of RNA were measured spectrophotometrically and RNA quality was determined by electrophoresis.

RT-PCR was performed using 100 ng RNA as the template with the Access RT-PCR system (Promega) in conjunction with non-degenerate primers designed directly from *C. gigas* sequence data.

Cgigas.cath1f: 5′-ggt tgc aat ggt aga tat cc-3′; Cgigas.cath1r: 5′-gaa ttg gcc atc agc cag ta-3′

The Promega Access RT-PCR system uses avian myeloblastosis virus (AMV) reverse transcriptase for first strand cDNA synthesis, and the thermostable *Tfl* DNA polymerase for second strand cDNA synthesis and amplification. First strand cDNA synthesis was performed with an initial 45-minute incubation at 48°C. Following inactivation of AMV-RT by a two minute incubation at 94°C, second strand synthesis, consisting of 40 cycles of 53°C (1 min), 68°C (2 min) and 94°C (30 sec) was performed. A final extension phase at 68°C (7 min) was followed by storage at 4°C. Following the initial RT-PCR, a nested PCR reaction was performed on 2 μl product using an annealing temperature of 56°C, 30 cycles and Taq polymerase (Promega) and a new forward primer again designed from specific *C. gigas* sequence.

Cgigas.cathnf: 5′-ctg aag gtg cct ggt ccc-3′; Cgigas.cath1r: 5′-gaa ttg gcc atc agc cag ta-3′

```
        801                                                    850
a       TGGGGACGGC TGTAATGGTG GCTATCCTGC TGAAGCTTGG AACTTCTGGA
b       .......... .......... .......... .......... ..........
c       TGGGGACGGC TGTAATGGTG GCTATCCCTC TGGAGCATGG AACTTCTGGA
d       TGGTGATGGT TGTCAAGGTG GATTTCCTGG TGTAGCATGG GACTATTGGG
e       TGGAGATGGA TGCAAGGGTG GATATCTGGG TCCGGCTTGG CAGTTCTGGG
f       TGGCGATGGT TGTGATGGAG GATATCCTAT CAGTGCGTGG CAATACTTCG
forward       GGC TGTAATGGTG GCTAT       (216 degeneracies)
primer          T   CC A   A  A
                A   G G

        1301                                                   1350
a       CTACTGGCTG GTTGCCAACT CCTGGAACAC TGACTGGGGT GACAATGGCT
b       CTACTGGCTG GTTGCCAACT CCTGGAACAC TGATTGGGGT GACAATGGCT
c       CTACTGGCTG GTTGCCAACT CTTGGAACCT TGACTGGGGT GATAATGGCT
d       TTACTGGTTG ATTGCCAATT CATGGAATGA AGATTGGGGT GAGAAGGGAC
e       GTACTGGCTG GTGGCCAACT CCTGGGGTGA CGATTGGGGA GACAATGGTT
f       TTACTGGCTT CTTGCAAATC AGTGGAACAG AGGCTGGGGC GATGACGGGT
reverse   CTGGCTG GTNGCCAACT C          (32 degeneracies)
primer          T A       T
```

Figure 1. Alignment of cathepsin B sequences from (a) *Homo sapiens*; (b) *Bos taurus*; (c) *Mus musculus*; (d) *Schistosoma japonicum*; (e) *Aedes aegypti*; and (f) *Triticum aestivum*. Conserved regions are boxed; primers designed from such regions are shown in bold.

Aliquots of the amplification reaction were analysed by agarose gel electrophoresis (1.5%) to check amplification efficiency.

Northern Blotting

For the probe, an XbaI and EcoRI (Promega) fragment containing the cathepsin B sequence was excised from the p*CG*cathI plasmid by restriction digest with *Xba*I and *Eco*RI (Promega). The cathepsin B fragment was purified by agarose gel electrophoresis and extraction from the gel was performed using the Qiaex extraction kit (Qiagen). 25 ng of this extracted DNA was used for probe construction using a Prime-It II random primer labelling kit (Stratagene) and ^{32}PdATP (Amersham).

RNA totalling up to 30–100μg from larvae one day PF and around settlement and from both adult digestive gland, adductor muscle and gonads and larvae at different stages of development were analyzed using Reliant RNA gels in MOPS buffer (FMC BioProducts). RNA was electrophoresed for two hours at 3.5 volts per cm. RNA markers (Promega) were run alongside the RNA samples so the size of the CathB transcript could ultimately be determined. Following electrophoresis the markers were stained with ethidium bromide. The RNA samples were then blotted onto Hybond N+ membrane and the position of the RNA markers recorded on the membrane which was then placed at 80°C for two hours to fix the RNA to the membrane.

The Hybond N+ membrane was pre-hybridized for one hour at 42°C in 10 ml ULTRAhyb™ ultrasensitive hybridisation buffer (Ambion). ^{32}P-labelled probe was added to the membrane which was left shaking at 42°C. Following hybridization for 16 hours, the probe was removed and the membrane was washed for 2 × 5 min in 2 × SSC, 0.1% SDS at 42°C and then for 1 × 10 min in 0.1 × SSC, 0.1% SDS at 42°C. The membrane was then sealed in Saran wrap and exposed to X-ray film for three days at –80°C.

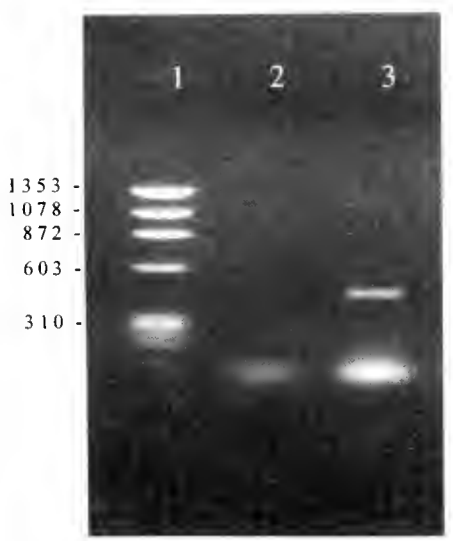

Figure 2. Amplification of cathepsin B fragment from adult *C. gigas* DNA using primers cathIf and cathIr. (PCR conditions are described in section 2.2). A total of 5 μl PCR products were analyzed on a 1.2% agarose gel. Lane 1 = ϕ×174/HaeIII size markers; lane 2 = –ve control lacking template DNA; lane 3 = template DNA extracted from adductor muscle. The sizes of the molecular weight markers are indicated in base pairs.

```
1GGT TGC AAT GGT AGA TAT CCT GAA GGT GCC TGG TCC CAA TTT AAG45
  G   C   N   G   R   Y   P   E   G   A   W   S   Q   F   K
46AGC AAG GGA CTC AGC TCA GGT TGG CTA TAT GGT GAT AAA AAG TAC90
  S   K   G   L   S   S   G   W   L   Y   G   D   K   K   Y
91TGC AAA GCT TAT AGT TTA CCT CCT TGC AGT CAT CAT GTA AAT GGT135
  C   K   A   Y   S   L   P   P   C   S   H   H   V   N   G
136TCT CAC CCT GCT TGC ACA GGC AGC ACT AGA ACT CCT GTC TGC ACA180
  S   H   P   A   C   T   G   S   T   R   T   P   V   C   T
181AAG GAA TGT GCC GCT GAA TCA GGC AGG ACT TAC AGT TCA GAC ATC225
  K   E   C   A   A   E   S   G   R   T   Y   S   S   D   L
226CGC CAC GGA AAA TCA GTC TAC TCC GTC AGA GGT GAG GCC AAC ATC270
  R   H   G   K   S   V   Y   S   V   R   G   E   A   N   L
271ATG CAT GAG ATT ATG ACC CGC GGA CCT ATG GAA GCT TCA TTC ACT315
  M   H   E   L   M   T   R   G   P   M   E   A   S   F   T
316GTT TAC CAA GAC TTT TTG GCT TAC AAA TCA GGA GTT TAC CAT CAT360
  V   Y   Q   D   F   L   A   Y   K   S   G   V   Y   H   H
361GTG ACC GGA AGT GCT CTT GGA GGT CAT GCT GTC AAA ATC ATG GGA415
  V   T   G   S   A   L   G   G   H   A   V   K   I   M   G
406TGG GGA GTA GAA AAT GGC ACT CCT TAC TGG CTG ATG GCC AAT TC450
  W   G   V   E   N   G   T   P   F   W   L   M   A   N
```

Figure 3. Nucleotide sequence and deduced amino-acid sequence of the cloned 450 b.p. cathepsin B fragment from *C. gigas*. Primer sequences are shown in bold.

RESULTS

Isolation of a C. gigas *Cathepsin B Clone*

Amplification of genomic DNA with degenerate primers designed from conserved regions of 3 vertebrate, 2 invertebrate and 1 plant cathepsin B nucleotide sequences (Fig. 1), resulted in amplification of a 450 bp fragment (Fig. 2).

Sequence Analysis of the C. gigas *Cathepsin B Fragment*

The nucleotide and deduced amino acid sequence of the cathepsin B clone are shown in Figure 3. As predicted from the PCR amplification, the cloned cathepsin B fragment was 450 bp long. The deduced amino acid sequence was compared with cathepsin B amino acid sequences for *Homo sapiens* (human), *Rattus norvegicus* (rat) and *Bos taurus* (cow) (Fig. 4). The *C. gigas* cathepsin B fragment showed 60% identity to *H. sapiens*, 59% identity to *R. norvegicus* and 58% identity to *B. taurus* at the amino acid level.

Expression Analysis of the Cathepsin B Gene in C. gigas

A cathepsin B transcript was detected in both adult and larval tissues after reverse transcription and two rounds of PCR (Fig. 5), indicating cathepsin B expression in both adult and larval tissues. For a quantitative but less sensitive technique, northern blot analysis was performed on a selection of adult tissue and larvae at different stages of development (Fig. 6). The probe identified a

```
C. gigas        GCNGRYPEGA WSQFKSKGLS SGWLYGDKKY CKAYSLPPCS HHVNGSHPAC
H. sapiens      GCNGGYPAEA WNFWTPKGLV SGGLYESHVG CRPYSIPPCE HHVNGSPPPC
R. Norvegicus   GCNGGYPSGA WNFWTPKGLV SGGVYNSHIG CLPYTIPPCE HHVNGSPPPC
B. taurus       GCNGGFPSGA WNFWTKKGLV SGGLYNSHVG CRPYSIPPCE HHVNGSRPPC

C. gigas        TGSTRTPVCT KECAAESGRT YSSDLPHGKS VYSVRGEANL -MHELMTRGP
H. sapiens      TGEGDTPKCS KICEPGYSPT YKQDKHYGYN SYSVSNSEKD IMAEIYKNGP
R. Norvegicus   TGEGDTPKCN KMCEAGYSTS YKEDKHYGYT SYSVSDSEKE IMAEIYKNGP
B. taurus       TGEGDTPKCS KTCEPGYSPS YKEDKHFGCS SYSVANNEKE IMAEIYKNGP

C. gigas        MEASFTVYQD FLAYKSGVYH HVTGSALGGH AVKIMGWGVE NGTPYWLMAN
H. sapiens      VEGAFSVYSD FLLYKSGVYQ HVTGEMMGGH AIRILGWGVE NGTPYWLVAN
R. Norvegicus   VEGAFTVFSD FLTYKSGVYK HEAGDVMGGH AIRILGWGIE NGVPYWLVAN
B. taurus       VEGAFSVYSD FLLYKSGVYQ HVSGEIMGGH AIRILGWGVE NGTPYWLVGN
```

Figure 4. Amino acid sequence comparison of cathepsin B from *C. gigas* with sequences from *Homo sapiens*, *Rattus norvegicus*, and *Bos taurus*. Amino acid residues that are identical to *C. gigas* cathepsin B are boxed.

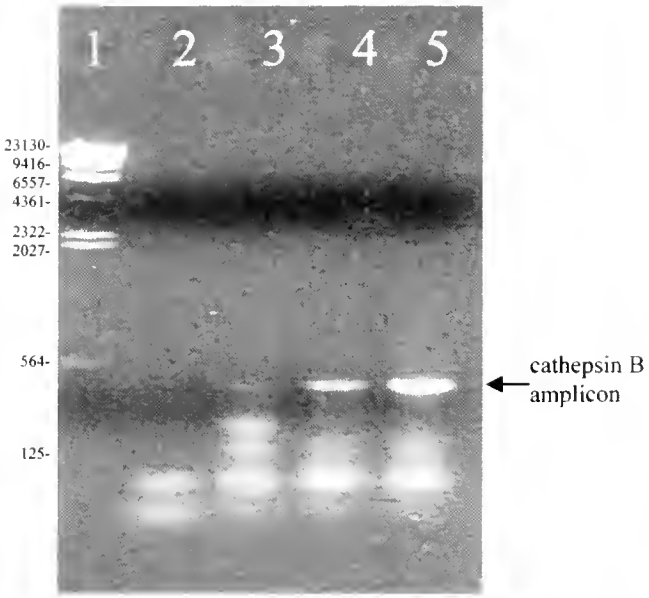

Figure 5. RT-PCR analysis of cathepsin B gene expression in *C. gigas*. Total RNA was reverse transcribed and submitted to two rounds of PCR as described previously. A total of 5 µl PCR products were analyzed on a 1.2% agarose gel. Lane 1 = λ*Hin*dIII markers; lane 2 = –ve control; lane 3 = 100 ng RNA from adult digestive gland; lane 4 = 100 ng RNA from larvae 2 days before settlement; lane 5 = 100 ng RNA from larvae 2 days post settlement.

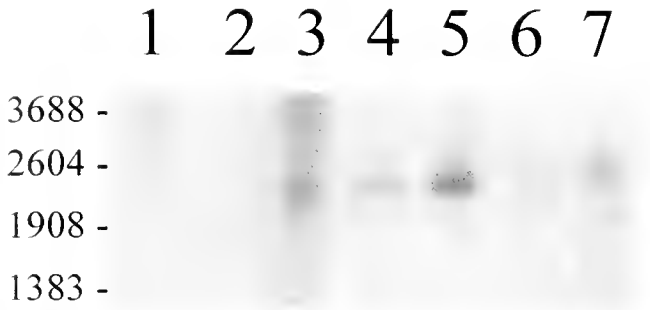

Figure 6. Northern blot analysis of *C. gigas* total RNA from adult and larval tissue. An [α-^{32}P]ATP-labelled 450-bp fragment of *C. gigas* cathepsin B DNA was hybridized with total RNA from adult and larval tissue. Lane 1 = 30 µg RNA from adult digestive gland; lane 2 = 30 µg RNA from adult adductor muscle; lane 3 = 30 µg RNA from adult gonads; lane 4 = 30 µg RNA from larvae 1 day post fertilization; lane 5 = 100 µg RNA from larvae 1 day post fertilization; lane 6 = 30 µg RNA from larvae 2 days before settlement; lane 7 = 30 µg RNA from larvae 2 days post settlement 2 µ l RNA markers (Promega) were run alongside RNA samples, the molecular weights were indicated.

cathepsin B transcript of approximately 2.4 kb in larval tissue and in adult gonad tissue. No expression was detectable in RNA extracted from adult digestive gland or adductor muscle.

DISCUSSION

To our knowledge, this is the first study to adopt molecular techniques to clone and partially sequence a gene encoding a the proteolytic enzyme cathepsin B in any shellfish. The amino acid sequence of cathepsin B in *Crassostrea gigas* showed 60, 59 and 58% identities to cathepsin B amino acid sequences in the human, cow and rat, respectively. Despite the obvious evolutionary distance between *C. gigas* and the three mammalian species, there is still a high degree of similarity between the oyster and mammalian cathepsin B sequences. It has been previously reported that bivalve cathepsins do share certain biochemical properties with mammalian cathepsins (Zeef & Dennison 1988). For example, the surf clam (*Spisula solidissima*) has been shown to possess a cathepsin with similar specificity and inhibition properties to mammalian cathepsin B (Chen & Zall 1986).

We detected cathepsin B gene expression in all developmental stages that we tested, reflecting the essential nature of proteolysis in repair, sanitation and metabolic regulation (Hawkins 1991, Ciechanover & Schwartz 1994). Northern blot analysis indicated that the level of cathepsin B expression was higher in larvae than in adult tissue; so much so that the level of cathepsin B expression was too low to be detected by northern blot analysis in adult digestive gland or adult adductor muscle tissue. This was consistent with our expectation of fast protein turnover during periods of rapid larval development (Fujii et al. 1991, Hawkins 1991). In adults, cathepsin B gene expression was only sufficient to be detected at the northern blot level in gonadal tissue. It is likely that this relatively high level of transcription occurred because the oysters had recently spawned, after which there is significant breakdown and reorganization of the remaining cells (Peek & Gabbott 1990).

In summary, we have developed a molecular probe to measure expression of the cathepsin B gene in *C. gigas*. We have detected expression of that cathepsin B gene throughout development, using RT-PCR. Findings indicate that the level of cathepsin B expression varied according to life stage, with higher levels of expression in larval tissue. Using this probe, our future work will relate expression to both the quantity (by western blotting) and activity (by biochemical assay) of cathepsin B, thus helping to resolve the mechanisms regulating cellular levels of this key proteolytic enzyme.

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IMPROVED METHOD FOR ROTAVIRUS DETECTION IN OYSTERS USING RT-PCR: SUITABILITY OF A COMMERCIAL PCR KIT

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ABSTRACT Commercially marketed kits are now available for PCR reactions. This study was conducted to determine the suitability of one of these kits (PCR supermix kit from Life Technologies Inc) for use in environmental testing for rotavirus in commercially cultured oysters. We focused on developing a rapid, efficient and inhibitor-free oyster-processing procedure which could be used for sensitive viral genome amplification by reverse transcripts PCR (RT-PCR) in raw oysters using a commercial kit for genome amplification. Rotavirus SA11 strain was used to evaluate the efficiency of virus recovery. Oyster tissues were seeded with 3×10^7 ffu of rotavirus. Oyster-processing included elution in TPB (Tryptone phosphate broth)/glycine (pH 9.0), virus precipitation using polyethylene glycol, sonication, and RNA extraction. RNA extraction methods evaluated included CTAB/SDS followed by phenol/chloroform extraction (standard method), or the commercial reagent TRIZOL LS™. Both were equally effective for removal of PCR inhibitors. Detection limit of the method described in this study was 0.03 ffu rotavirus SA11 recovered from the oyster tissues. We conclude that the Supermix® commercial PCR kit can provide a more rapid and sensitive alternative for virus detection in oysters when compared to traditional PCR protocols. This is especially beneficial when large numbers of environmental samples require analysis.

KEY WORDS: oysters, RT-PCR, PCR supermix® kit, rotavirus

INTRODUCTION

Enteric virus transmission through consumption of fecally-contaminated shellfish is a significant public health concern (Richards 1985, Jaykus et al. 1994). Fecal coliforms have long been considered the best, if imperfect, indicators of fecal pollution. However, these indicators have come under increasing scrutiny as acceptable indicators of viral health hazard in commercial shellfish, and waters used for their cultivation (CDC, 1991). The primary arguments against use of fecal coliforms as indicators of fecal pollution are:

1. Shellfish and waters with acceptable levels of fecal coliforms may still be contaminated with enteric viruses.
2. Depuration methods for removal of bacterial pathogens from shellfish have not consistently removed viruses.
3. Depurated shellfish have been proven a source of outbreaks of viral-induced gastroenteritis (Sobsey et al. 1987, Caul et al. 1993).

In addition, Abad et al. (1997) demonstrated that pathogenic viruses could be detected in mussels from areas regarded as unpolluted, safe for swimming, and suitable for harvesting shellfish using standard bacterial indicators. In the absence of effective indicators, investigators have attempted the direct detection of viruses from shellfish and their cultivation waters. Shellfish are readily contaminated with viruses present in sewage contaminated waters due to the concentration effect of filter feeding (Larkin & Hunt 1982, Gerba 1988, Cromeans et al. 1997, Barardi et al. 2001). Since viruses do not replicate in shellfish tissues, the vector potential of shellfish is thought to be due to the stability of viruses in their tissues. The risk of acquiring a shellfish-borne viral disease is substantial as they are often eaten raw, including the intestinal tract (Heller et al. 1986, Wanke & Guerrant 1987, Speirs et al. 1987, Bouchriti & Goyal 1993, Kohn et al. 1995). In addition, cooking experiments performed with artificially contaminated mussels revealed that five minutes after the opening of the mussel valves, rotaviruses and hepatitis A virus could still be recovered in steamed shellfish. Under commercial depuration conditions, health-significant enteric viruses, such rotavirus and hepatitis A virus, could be recovered from bivalves after 96 hours of immersion in a continuous flow of ozonated marine water (Abad et al. 1997).

Rotaviruses represent 80% of recognized viral etiologies, and 140 million cases of diarrhea per year worldwide. Structurally, rotaviruses belong to the "naked" class of viruses. These are generally more environmentally resistant than enveloped viruses (Gerba et al. 1996). They are commonly found in wastewater and in addition can be concentrated by shellfish. Thus, the environment constitutes a significant reservoir for the virus (Bajolet & Chippaux-Hyppolite 1998). Examination of shellfish for viral contamination has generally relied on cell culture (Berg et al. 1984, Cromeans et al. 1997, Suñén & Sobsey 1999). However, wild-type enteric viruses do not readily grow in cell culture. They also generally require long periods of adaptation and specific antigen assays for virus detection, since no visible cytopathic effect is produced in vitro. Polymerase chain reaction (PCR) can be used to enzymatically amplify nucleic acid sequences present in low copy numbers in environment samples to detectable levels. The speed, specificity, low cost, and ease of use of PCR have led to its increasing use in environmental science (Metcalf et al. 1995, Abbaszadegan et al. 1999). Despite the sensitivity of PCR reactions, interfering substances in the environmental samples can severely limit the usefulness of this approach (Schwab et al. 1993, Atmar et al. 1993, Shieh et al. 1995).

The overall objective of this study was to evaluate and establish a new, molecular-based protocol to routinely monitor shellfish for infectious viruses, using rotavirus SA11 and the oyster *Crassostrea gigas* as a virus-shellfish model system. We evaluated a simple protocol for oyster extract preparation, and a cost-effective method for viral RNA isolation from these extracts. After cDNA

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synthesis, the strategy was to use a PCR kit together with specific primers capable of rapidly identifying the presence of infectious viral pathogens in environmental samples through genome amplification with the accuracy and speed required for routine shellfish monitoring by laboratories.

Rotaviruses were chosen as a model to develop this assay as they are responsible for severe gastroenteritis in humans and animals. They have been implicated in outbreaks of waterborne gastroenteritis in many countries (Ansari et al. 1991). After replicating in the gastrointestinal tract, they are excreted and may be dispersed in environmental waters (Kapikian & Chanock 1996, Hart & Cunliffe 1997). The stability of human rotaviruses in environmental waters, and their resistance to physico-chemical treatment processes in sewage treatment plants may facilitate their transmission (Hurst & Gerba 1980). In addition, their presence in drinking water, (Deetz et al. 1984, Strappe 1991) sea water, (Goyal & Gerba 1983) and shellfish (Lewis & Metcalf 1988, Hafliger et al. 1997) has been described.

MATERIALS AND METHODS

Virus and Cells

Simian rotavirus SA11 (group A, simian serotype G3) was propagated and assayed by indirect immunofluorescence in MA104 cells, a continuous line of fetal Rhesus monkey kidney. Cells were cultivated in Eagle's minimal medium (MEM) supplemented with 1X non-essential aminoacids, 15 mM Hepes buffer, 2 mM glutamine and 10% (v/v) fetal calf serum (Birch et al. 1983). Infected fluid was titrated by indirect immunofluorescence assay as described by Barardi et al. (1998) using 10 μg/ml of trypsin in the maintenance medium (as described above without Fetal Calf Serum).

Experimental Design for Viral Recovery Studies

Oyster Inoculation

Oysters (*Crassostrea gigas*) were obtained from a cultivated system oyster farm in Florianópolis City, Santa Catarina State, Brazil. Oyster shells were scrubbed with a stiff brush in running potable water (ca. 0.7 ppm free chlorine). Shell surfaces were then coated with a 70% ethanol solution and left to air dry for 30 minutes at room temperature in a biological safety cabinet. Shells were then opened at the hinge with an autoclaved oyster knife. Each oyster was seeded with 300 μl of rotavirus-infected tissue culture supernatant containing 3×10^7 focus forming units (ffu) using a micropipette tip inserted at three points of the visceral area as described previously (Cromeans et al. 1997, Barardi et al. 1999). After adsorption for 30 minutes at room temperature, the oyster extract was prepared (see later in text). Negative controls consisted of washed and disinfected shells from non-seeded oysters from the same source.

Oyster Extract Preparation

The following method for preparation of virus extracts from oysters was examined for effectiveness of viral recovery for the subsequent RT-PCR assay (Barardi et al. 1999 with minor modifications): 10–20g of oysters flesh were transferred to a sterile Schott® bottle containing 100 ml of pre-chilled (ice bucket) 10% (v/v) tryptose phosphate broth (TPB) (100% TPB contains 20g tryptose, 2.0g glucose, 5.0g NaCl and 2.5g Na_2HPO_4, pH 9.0) prepared in 0.05M glycine (pH adjusted to 9.0 using 2N NaOH). Tissues were then homogenized with a shaft blender (Ultra-Turrax T-25 Ika®) at 24,000 rpm for 30s. The resulting homogenate was placed in a 50 ml centrifuge tube, shaken at 250 rpm for 30 min. at 22°C, and centrifuged at 10,000 Xg for 30 min at 4°C. The pellet was discarded and supernatant pH adjusted to 7.5 using 2M HCl. Polyethylene glycol solution (PEG, MW = 8000) (50%, w/v) prepared in 10% TPB was added to a final concentration of 8% (w/v). The mixture was stirred for 2 h at 4°C, then centrifuged at 10,000 ×*g* for 20 min a 4°C. The supernatant was discarded and the pellet resuspended in 5.0 ml of 0.15 M Na_2HPO_4, pH 9.0. The resuspended pellet was then sonicated at high power twice for 30s and transferred to a fresh centrifuge plastic tube and the pH readjusted to 7.4 using 2M HCl. This preparation was designated oyster extract B (Fig. 1).

To study the effectiveness of this virus extraction method using both PCR and RT-PCR, unseeded oysters were extracted using the same procedure. These oyster extracts were then seeded with the same amount of virus used for preparation of spiked-whole-oyster extracts. Seeded extracts were designated as positive controls-A (Fig. 1).

Viral RNA Extraction from Oysters Extracts

Two techniques of viral RNA extraction were compared for their effectiveness in detecting Rotavirus genome from oyster extracts by RT-PCR. **Technique I** (Barardi et al. 1999): An aliquot (0.5 ml) of each oyster extract: A (positive control) and B (experimental extract) (equivalent to 2.5×10^7 ffu) were used for viral RNA isolation. An equal volume of trichlorotrifluoroethane (Aldrich) was added to remove lipids from each aqueous oyster extract, and the suspensions centrifuged at 6,000 rpm in a microcentrifuge (Eppendorf®) for five minutes at room temperature. The aqueous phase was transferred to another sterile Eppendorf® tube. Tris-HCl (pH 7.5), EDTA, SDS and proteinase K were added at final concentrations of 10 mM, 5 mM, 0.5% (w/v) and 400 μg/ml, respectively. Samples were then incubated at 37°C for 30 min. Cetyltri-

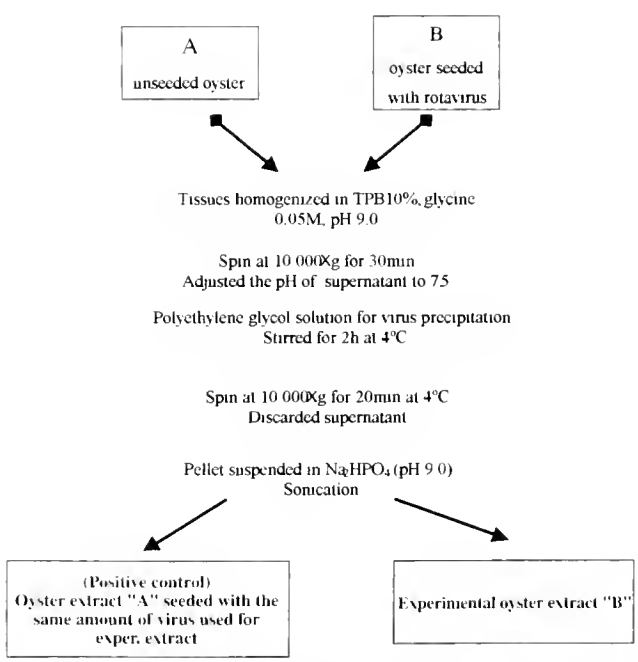

Figure 1. Outline of techniques used to prepare oysters extracts A, and B prior to detection using RT-PCR.

methylammonium bromide (CTAB) and NaCl were added to final concentrations of 1.3% (w/v) and 0.52 M, respectively, and samples incubated at 56°C for 30 min. Samples were subsequently extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The aqueous phase was transferred to another microfuge tube and an equal volume of chloroform added. The aqueous phase was then precipitated in 3 vol of chilled 100% ethanol at −20°C. Resulting pellets were washed with chilled 70% (v/v) ethanol, suspended in 50 µl of Milli-Q water, and stored at −20°C for RT-PCR assays. **Technique II**: An aliquot (0.5 ml) of oyster extracts (A and B) was used for viral RNA isolation. According to manufacturer specifications, each sample was added to 1.5 ml of TRIZOL LS™ reagent and residual cells in the sample lysed by passing the suspension several times through a blue tip form P-1000 micropipette. Samples were incubated for five minutes at room temperature, 0.4 ml of chloroform added, and the sealed tube shaken vigorously by hand for 15 seconds. Samples were then incubated at room temperature for 10 minutes followed by centrifugation at 12,000 ×*g* for 15 minutes at 28°C. The aqueous phase was transferred to a fresh tube and 0.5 ml of isopropyl alcohol added, followed by incubation at room temperature for 10 minutes and centrifugation (12,000 ×*g*, 10 min, 4°C). The supernatant was removed and pellet washed once with 1 ml, 75% ethanol. At the conclusion of the procedure the RNA pellet was air dried, suspended in 50 µl of Milli-Q water, and stored at −80°C for RT assays.

RT-PCR Assays

The level of rotavirus detection by RT-PCR was determined for the extracts A (positive control) and B (experimental extract) using 2-fold serial dilutions of viral RNA ranging from 1:5 to 1: 5,000,00 and corresponding, respectively, to 2×10^4 to 0.015 ffu. **Primers:** the oligonucleotide primers BEG9 (5′-GGC TTT AAA AGA GAG AAT TTC CGT CTG G-3′) and END9 (5′-GGT CAC ATC ATA CAA TTC TAA TCT AAG-3′) (Gouvea et al. 1990), were used for reverse transcription and first PCR. These primers produce full-length copies of gene 9 (1062 base pairs) from any group A rotavirus strain. The primers ET3 (5′-CGT TTG AAG AAG TTG CAA CAG-3′) and END9 were used for the serotype-specific semi-nested PCR (second amplification-374 base pairs) (Gouvea et al. 1990), as the SA11 simian rotavirus, which belongs to serotype G3, has been used as a model for group A rotaviruses in this study.

Two RT-PCR protocols were compared for their sensitivity to detection of the lowest quantity of viral RNA. **Protocol 1:** (Barardi et al. 1999 with minor modifications). 5.0 µl aliquots of diluted RNA isolated either by technique I or II (in separate reactions) and 200 pmol BEG9 and END9 primers were heated at 99°C for 5 min, followed by chilling on ice for 1 min. The denatured RNA was added to the reaction mix consisting of 20 mM Tris-Cl, pH 8.4, 50 mM KCl, 0.4 mM each dATP, dCTP, dTTP and dGTP, 1.5 mM $MgCl_2$, 10 mM DTT and 40 units M-MLV reverse transcriptase (GibcoBRL). Reverse transcription of viral genomic RNA was carried out at 37°C for 90 min After this step, 5 units *Taq* DNA polymerase were added, and tubes overlayed with mineral oil. PCR amplification was carried-out for 25 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 3 min, and a final 10 min incubation at 72°C. For semi-nested PCR, 2.0 µl of the first PCR product was added to the reaction mix of 50 µl containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.2 mM each dATP, dCTP, dTTP and dGTP, 3.0 mM $MgCl_2$, 100 pmol ET3 and END9 primers, and 5 units *Taq* DNA polymerase. Tubes were then overlayed with mineral oil and the same PCR program used. **Protocol 2:** The PCR Supermix® kit (GibcoBRL) was used in this protocol. The manufacturer's protocol was followed, except for the concentration of $MgCl_2$ (see later in text). Five µl aliquots of diluted RNA isolated either by technique I or II (in separate reactions) were heated at 99°C for 5 min, then chilled on ice for 1 min. The denatured RNA was added to the reaction mix consisting of 50mM Tris-HCl, pH 8.3, 75 mM KCl, 3.0 mM $MgCl_2$, 0.5 mM each dATP, dCTP, dTTP and dGTP, 5 mM DTT, 100 pmol BEG9 and END9 and 40 units M-MLV reverse transcriptase. Reverse transcription of viral genomic RNA was carried out at 37°C for 90 min. After this step, 2.0 µl of reverse transcriptase product and 100 pmol BEG9 and END9 were added to 45 µl of the commercial reaction mix containing 22 mM Tris-HCl, pH 8.4, 55 mM KCl, 1.65 mM $MgCl_2$, 0.22 mM each dNTP and 22 units/ml *Taq* DNA polymerase. Additional $MgCl_2$ was added to the reaction mix to achieve a final concentration of 3.0 mM. Tubes were overlayed with mineral oil and heated at 80°C

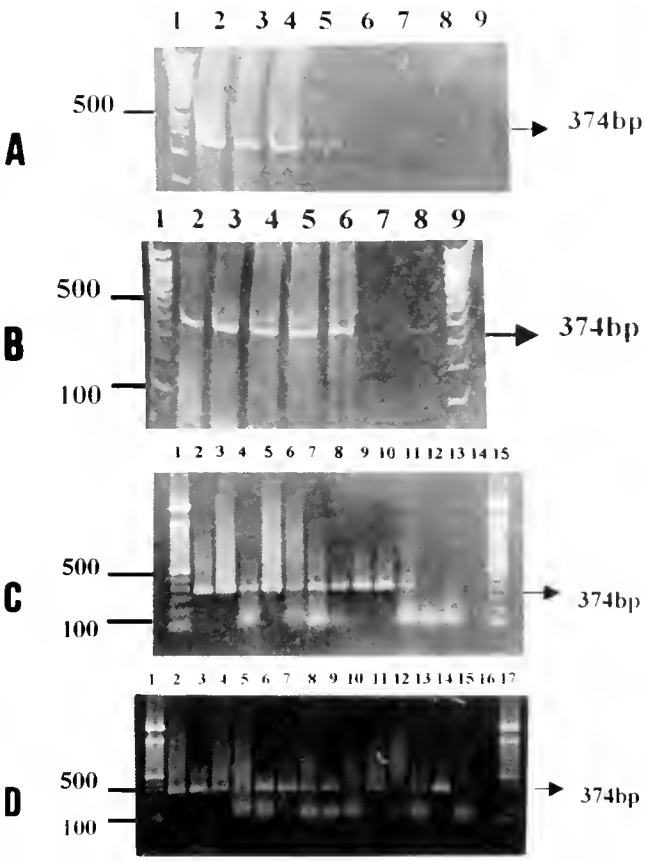

Figure 2. Semi-nested PCR products (A and B-traditional PCR and C and D-Supermix® kit) from simian rotavirus RNA extracted from oysters extracts A and B. (A) A extract (positive control). Lane 1: molecular weight marker; lanes 2 to 8: serial dilutions of viral RNA corresponding from 2×10^4 to 312 ffu respectively; lane 9: negative control. (B) B extract (exp. extract). Lanes 1 and 9: molecular weight marker; lanes 2 to 8: serial dilutions of viral RNA corresponding from 1.6×10^5 to 625 ffu respectively. (C) B extract (exp. extract). Lanes 1 and 15, molecular weight marker, lanes 2 to 13: serial dilutions of viral RNA corresponding from 1×10^5 to 0.07 ffu, lane 14: negative control. (D) A extract (positive control). Lanes 1 and 17: molecular weight marker; lanes 2 to 15: serial dilutions of viral RNA corresponding from 1×10^5 to 0.015 ffu, lane 16: negative control.

TABLE 1.

Sensitivity of RT-PCR for rotavirus detection using both PCR protocols with the different oyster extracts.

	Limit of Viral Detection (ffu)	
Oyster Extracts	PCR Traditional	PCR Supermix Kit
A (positive control)	312	0.03
B (experimental test)	625	0.15

for 1 min. The same PCR program described above was then used. PCR products were analyzed by electrophoresis in 1.5% (w/v) agarose, or 10% (w/v) polyacrylamide gels. Both were stained with ethidium bromide at 10 μg/ml, and visualized under UV light. Either nucleic acids isolated from non-seeded oyster extracts or just distilled water were used as negative controls for the RT-PCR reactions.

RESULTS

Sensitivity of Rotavirus Detection Using RT-PCR Assays

The sensitivity of two RT-PCR assays for virus detection was evaluated using total RNA isolated from oyster extracts A (positive control) and B (experimental extract). The RNA from both extracts were isolated according to Techniques I and II. Both techniques were equally effective for RNA isolation for the RT-PCR reactions (not shown). The minimum amount of virus (ffu) that could be detected in the second PCR (semi-nested) using the traditional PCR protocol (protocol 1) was 312 ffu for extract A (positive control) and 625 ffu for extract B (experimental extract). PCR Supermix® kit (protocol 2) was also used for oyster extracts A and B In this case, the minimal amount of virus (ffu) that could be detected in the second PCR (semi-nested) was 0.03 ffu for extract A (positive control) and 0.15 ffu for extract B(exp. extract). These results can be seen in Figure 2. Table 1 summarizes the sensitivity of RT-PCR using both PCR protocols.

DISCUSSION

Nested or semi-nested RT-PCR systems are among the most sensitive molecular biological detection methods available today, with the ability for detection of as little as a single copy of a viral genome (Hafliger et al. 1997). This paper describes the evaluation and establishment of a rapid and reliable protocol for virus detection in artificially-inoculated oysters using a commercial PCR kit for genome amplification. Rotavirus SA11 and the oyster *Crassostrea gigas* were used as a model system to evaluate the efficacy of two viral RNA isolation techniques, and the sensitivity of subsequent virus detection using two different RT-PCR methods. Two procedures were compared for purification of viral RNA from artificially infected oyster extracts. There was no relevant difference in rotavirus detection by RT-PCR between the two methods. TRIZOL LS™ reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, followed by chloroform proved to be effective for viral RNA purification. So, only results obtained with the less expensive method for nucleic acid extraction (phenol-chlorophorm) were presented, using two different RT-PCR protocols. Comparison of results obtained by RT-PCR, followed by semi-nested PCR detection, had confirmed that when the PCR Supermix® (a ready-to-use mixture of recombinant *Taq* DNA polymerase, salts, magnesium, and deoxyribonucleotide triphosphates) is used, the limit of virus detection was 0.03 ffu for the positive control (A), and 0.15 ffu for the experimental extract (B). In both cases, this was 4,000 to 10,000 times more sensitive when compared with the virus detection limit using the traditional PCR protocol where all reagents were added separately.

This study demonstrates improvements and simplification of a highly sensitive and simple method to prepare oyster tissue extracts, and application of a specific and sensitive RT-PCR based system for the detection of rotavirus. We anticipate that the methods described here will be further improved and optimized in a single multiplex PCR reaction for detection of a greater variety of human enteric viruses commonly found contaminating shellfish and aquatic environments.

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POPULATION STRUCTURE AND DEMOGRAPHY OF THE PUELCHE OYSTER (*OSTREA PUELCHANA*, D'ORBIGNY, 1841) GROUNDS IN NORTHERN PATAGONIA, ARGENTINA

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ABSTRACT The three main grounds of the flat oyster (*Ostrea puelchana*, D'Orb.) are located at the northwestern portion of the San Matías Gulf, at Northern Patagonia, Argentina. The population structure and demography of the grounds at Banco Reparo, Las Grutas and Bajo Oliveira were studied through several diving and dredge surveys performed between 1985 and 1991. The complete population of the grounds located at Banco Reparo and Bajo Oliveira, and the population of the central part of Las Grutas ground, were assessed, measuring oyster density and spatial distribution, size structure, proportion of clustered and free oysters, proportion of oysters carrying epibiotic males, and oyster's life habit. The puelche oyster is distributed almost continuously along the northern coastal area of the San Matías Gulf, at depths ranging from two to 20 minutes. The grounds are likely to be interconnected by larval dispersion. The three studied grounds differed in their demographic characteristics, and it was difficult to compare biomass between the grounds due to different methods of assessment. However, the results enabled us to conclude that high oyster density areas were always limited on any of the grounds, with maximal densities only on relatively small patches. Patches of recruits were never found in the surveys, suggesting that a clear spatial segregation of settlement does not occur in this species. Oyster size structure at Banco Reparo and Las Grutas was similar, while individuals at the Bajo Oliveira population were very large, with mean population size 20 mm larger than on the other two grounds. Size classes below 45 mm were always poorly represented or absent on the three grounds. The portion of the population comprising of individuals <30mm was represented by the epibiotic males who represented a "hidden" mode. The three populations differ in their dynamics, mainly in the turnover rates of individuals. At Bajo Oliveira the medium size individuals are absent (there is a hiatus between the two modes) and the mean size of adult oysters is the largest (89 mm). In contrast, at Las Grutas, the intermediate sizes between the two modes are present (there is no hiatus between the two modes) and the mean size of adults is the smallest (66 mm). Banco Reparo occupies an intermediate position. Based on this information we suggest that turnover rate is then maximal at Las Grutas, intermediate at Banco Reparo, and minimal at Bajo Oliveira. The puelche oyster grounds at the San Matías Gulf offered the rare opportunity of studying the structure of one of the few natural and untouched flat oyster populations in the world. Work helped support the closure of the fishery as a policy mainly based on the vulnerability of the stocks.

KEY WORDS: oysters, *Ostrea puelchana*, Patagonia, natural grounds, demography, density distribution

INTRODUCTION

Ostrea puelchana (d'Orbigny 1841), commonly named "puelche" or "Patagonian", is a flat oyster belonging to the Family Ostreidae (Subfamily Ostreinae) (Stenzel 1971). This species is distributed from Rio Grande do Sul (30°S, 50 W, Brasil) to Bahía Camarones, Northern Patagonia (44°S, 66°W, Castellanos 1957) (Fig. 1). Dense aggregations ("grounds") have been reported only at the southern limit of their distribution (Valette 1929, Castellanos 1957, Fernández Castro 1987, Pascual & Bocca 1988, Pascual 1993). However, there has been recent evidence of the presence of the species in deep waters (30–35 m depth) off the State of Santa Catarina, Brasil (Carlos Polis & Jaime Ferreira, pers. comm.). The distributional area of *O. puelchana* should then be extended northwards. Isolated individuals regularly appear along the northern Argentinean coast as by-catch in the mussel dredge fishery off Buenos Aires shores (M. Lasta, pers. comm.), and at intertidal sites of the same area. The occasional appearance of flat oysters has been reported for several localities along the coast of Buenos Aires Province (35–40 S; 57–62 W).

The presence of this species in Argentina was first reported by D'Orbigny (1841) who, as indicated by Castellanos (1957) misidentified this species as *Ostrea spreta*, a non commercial flat oyster that cohabits with *O. puelchana* at shallow coastal waters of the San Matías Gulf (40°40'S; 63°30'W) (Fig. 1). *O. spreta* is a small oyster, pink to purple colored, that attains a maximal size of 50 mm and forms beds inside San Antonio Bay (Fig. 1), in environments of strong tidal currents. Extreme types of both species may be easily recognized but a wide gradient of morphological characteristics makes the identification of young individuals difficult.

Ostrea puelchana has solid shells, lamellated and subnacreous, circular or subsquare. The upper or right shell is flat and lamellated. The lower or left shell is bigger, lamellated and convex (Castellanos 1957). The individuals have a diverse coloration, the juveniles are homogeneously cinnamon colored but frequently showing shells with longitudinal purple estriae. The adult specimens loose their cinnamon color and show yellow greenish or gray shells.

The puelche oyster lives isolated or in clusters. A cluster is formed when the larvae settle and grow on the shells of a "founder" oyster. The maximal size registered in this species is 140 mm of total height (TH: largest diameter from the umbo to the opposite margin). This size is rare, however, with size generally up to 120 mm in deep populations. Age is difficult to assess in this species; attempts at age revelation from readings on shell cuts have been unsuccessful. Nevertheless, external characters such as the thickness of the ligament and the rate of infection by perforating sponges, *Cliona celata* or the Mitylid *Litophaga patagonica*, suggest that it is a long-lived species.

The structure of some of the puelche oyster populations of the San Matías Gulf has been studied for the last 20 years. Most of this information is contained in unpublished technical reports (Vacas 1978, Vacas 1979), a thesis (Pascual 1993), and some has been published (Morriconi & Calvo 1989). A great amount of research effort was dedicated to the sexuality of this species (Calvo & Morriconi 1978, Morriconi & Calvo 1979, Fernández Castro 1987, Fernández Castro & Lucas 1987, Pascual et al. 1989, Pascual &

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Zampatti 1995, Pascual 2000). *O. puelchana*, while fitting the general reproductive pattern of brooding Ostreas, shows an alternative breeding system that makes it unique among Ostreids. In natural grounds, oysters change their sex to the female phase when they reach a shell size of 50–55 mm. At this point, the oyster acquires the ability of "carrying" small conspecific individuals settled on an expansion ("platform") of the anterior margin of the concave shell (Calvo & Morriconi 1978). These epibiotic oysters are always males during the reproductive season and their gonads mature synchronously with the gonads of its female "carrier" (Calvo & Morriconi 1978). The epibiotic males have their growth rate severely reduced by the influence of the female carrier (Pascual et al. 1989). This "dwarf" condition assures the persistence of each couple (carrier oyster + epibiotic male) for long periods of the carrier's lifetime.

The populations of the puelche oyster of the San Matías Gulf have been protected by a legal fishing closure established in 1971. This rare legal interdiction has been based for many years on the assumption of the vulnerability of the stocks. However, it was not before 1985 that a thorough sampling of the oyster grounds was initiated through the San Matías Gulf Oyster Project. This project was intended to gain knowledge on the demographic structure and spatial extent of the puelche oyster stocks, and on the reproductive ecology of the species. Simultaneously, an Oyster Culture Project has been developed.

The present paper presents information obtained from extensive surveys performed between 1985 and 1991 on the population structure and dimensions of the three main oyster grounds of the NW San Matías Gulf: Banco Reparo (BR), Las Grutas (LG), and Bajo Oliveira (BO) (Fig. 1).

MATERIALS AND METHODS

Study Site and Background Information

The Banco Reparo oyster ground is located in the NW San Matías Gulf (40°40′S; 63°30′W) (Fig. 1). The bottom is sandy, partially covered by pebbles and mollusk shells. Currents are strong (26–52 cm s^{-1}) and depth ranges from 2–3 m at low tide. Tidal range is 7.62 m in spring, and 5 m during mean tides (Servicio de Hidrografía Naval, 1969). Seawater temperature ranges from 7°C (August) to 23.5°C (January); and salinity ranges from 34–36‰ (Fernández 1989). The highest values of *chlorophyll a* are reached in March (23 mg l^{-1}), while the maximal values of phosphates and nitrites/nitrates are reported in spring (0.13 μg l^{-1}, and 9.25 μg l^{-1} respectively) (Fernández 1989). Dissolved oxygen fluctuates seasonally reaching its maximal value in August (7.69 mg l^{-1}) (Fernández 1989).

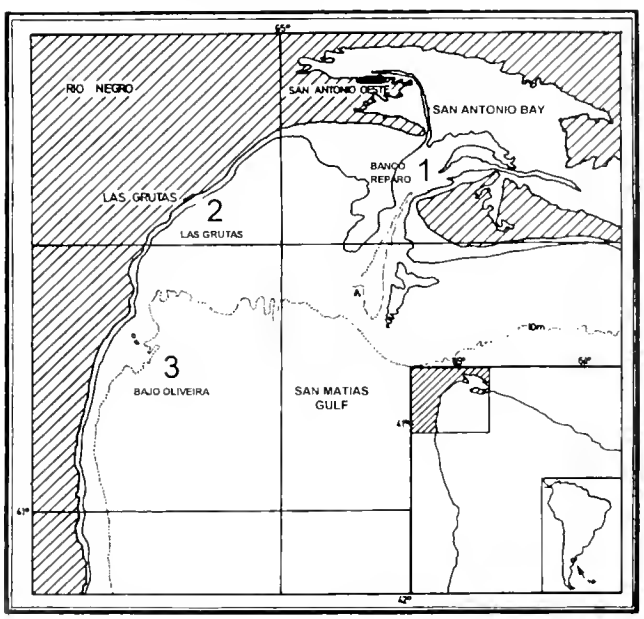

Figure 1. Location of the three main oyster grounds along the San Matías Gulf coast, Northern Patagonia (1 = Banco Reparo; 2 = Las Grutas, and 3 = Bajo Oliveira).

The Las Grutas oyster ground is located on open waters on the NW coast of the gulf, 15 km south from Banco Reparo (40°48′S; 65°05′W, Fig. 1 and Fig. 3). The bottom is composed of coarse sand and shell, occasionally interrupted by limestone platforms crossed by channels filled with sand. Tidal currents are weaker than in Banco Reparo (20–30 cm s^{-1}) and depth ranges from 2.5 to 6 m at low tide. Oysters live on sandy flats, on limestone platforms, and among dense stands of the macroalgae *Codium* sp. Water temperature ranges from 8°C (August) to 21°C (January), and salinity between 34 and 35‰ (Fernández 1989). The highest values of *chlorophyll a* were reported for March (42 mg l^{-1}, in 1987). Phosphates peak in April (10.5 μg l^{-1}), and nitrates/nitrites peak in March (13.5 μg l^{-1}). Dissolved oxygen shows a marked seasonality, reaching its winter peak on August (6.8 ml l^{-1}) (Fernández 1989).

The Bajo Oliveira oyster ground is located south from Las Grutas ground, also in the NW of the gulf (40°50′S; Figs. 1 and 3). The bottom is predominantly sandy and flat, covered by pebbles, small shell pieces and mollusk shells. Depth ranges from 4–22 m at low tide. Available oceanographic information is scarce and was gathered during a two survey conducted during 1971 and 1991 over the entire gulf, both performed in autumn, during which only one or two stations were located at Bajo Oliveira (Carreto & Verona 1971, Estéves et al. 1991). During those surveys salinity ranged from 33.9–34.9‰, *chlorophyll a* from 0.7–1.8 mg l^{-1}, nitrates were 0.78 μg l^{-1}, phosphates were 1.33 μg l^{-1} and water temperature was 17°C.

Sampling Design

At Banco Reparo, an area 1853 m long and 400 m wide, corresponding to the surface actually occupied by oysters, was positioned, on the basis of land references, and marked by buoys. A rectangular grid, comprising of 18, 2-m wide transects, each separated by a 100 m distance, was designed. Once each transect was positioned and marked, all the oysters found in each linear segment (10 × 1 m) at one side of the first sub-transect were collected by divers. The procedure was then repeated in the opposite direction. Thus, 20 sampling units were obtained on each sub-transect, and 80, as a maximum, over the entire transect. The eastern limit of each transect was determined by the absence of oysters along a distance of 100 m. The described procedure was performed 18 times and took 10 working days, from 23 October to 12 November 1985. The number of free oysters and clustered oysters was registered in each sample. All the oysters visible to the naked eye and one out of ten samples were collected and sized (total height in mm). Density data (oysters 10 m^{-2}) were used to produce a map of the population density distribution at the ground, this was used to estimate the area with different oyster densities. The area of the sectors included between isopleths was estimated by graphic computer software.

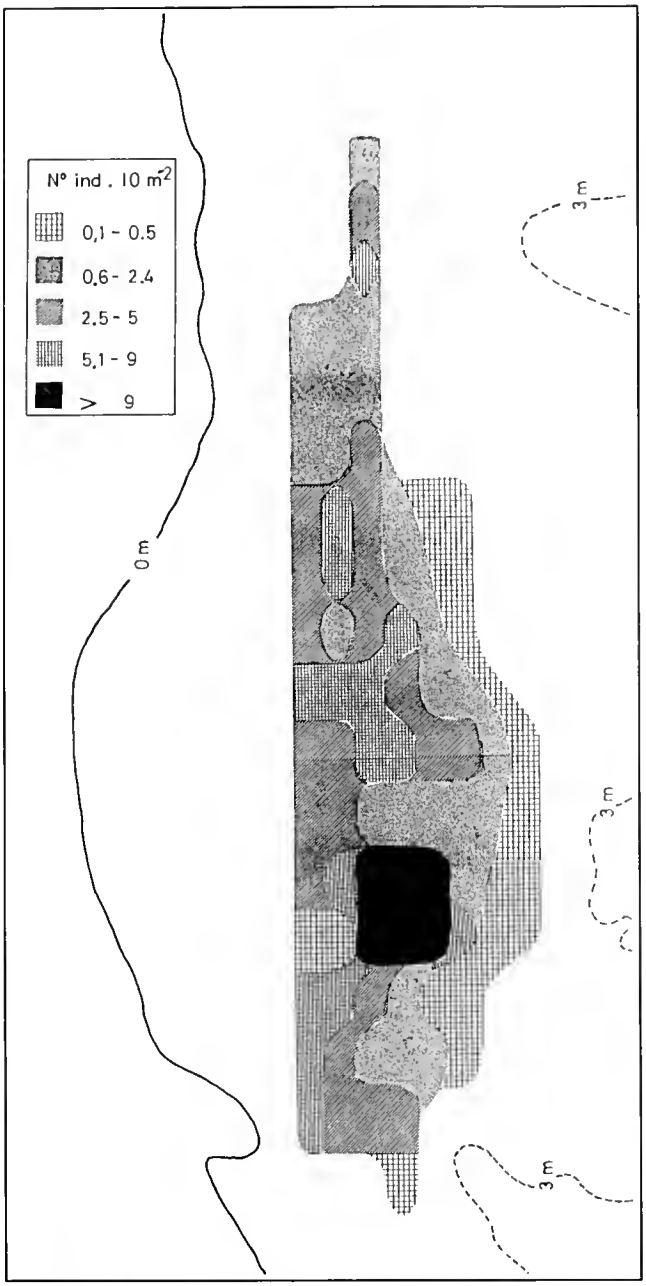

Figure 2. Distribution and abundance of oysters in Banco Reparo. Maximal densities are concentrated in the black stratum. Densities are number of oysters 10 m^{-2}.

Complementarily, sampling was performed to assess the predominant life position of individuals of different size ranges. Two transects, located in the center of the ground, were selected, and the divers registered, for each individual present along the two transects, its size and life position (oysters lying on its flat or on its concave shell). Oysters were allocated into three size groups: small (<3 cm); medium (3–7 cm) and large (>7 cm).

Sampling of oyster population structure at Banco Reparo was repeated October 1986 and November 1987 in the areas identified as high oyster density spots during the 1985 survey. A sample of all the oysters present in a 100 m transect was collected. Oysters from the samples were measured (total height: TH). The number and size of epibiotic (dwarf) oysters per carrier oyster were registered.

At Las Grutas only the central part of the ground, which was determined by previous sampling to have the highest density (marked by a star in Fig. 3), was prospected to describe the population structure. A station was fixed by a marking buoy and acted as a starting point from which seven radially spreading transects were laid out. Five to seven sampling units (1 m^2) were collected along each transect, at 15–20 m intervals. All the oysters within the frame were collected. The survey was completed between 16 to 28 November 1987.

The sampled oysters were separated as free and clustered oysters; they were measured (total height in mm) and counted. The epibiotic oysters fixed on the platform of carrier oysters (previously sacrificed) were also counted and measured.

Following the same procedure as in Banco Reparo, life position of oysters was also evaluated by diver collection of all oysters along a 200 m transect. Oysters lying on their flat shell or concave shell were collected in separate bags.

The Bajo Oliveira oyster ground, due to its great extent, had to be surveyed with a fishing research vessel equipped with a commercial dredge (mouth width: 2.5 m). Two surveys were conducted in June 1987 and May 1988. The first was the more detailed

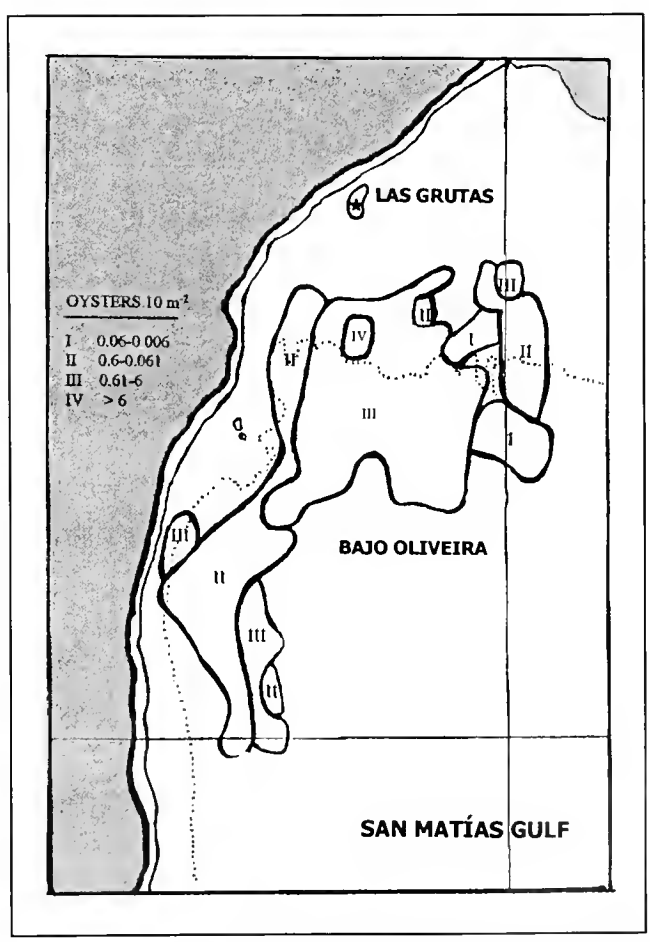

Figure 3. Oyster grounds at Las Grutas (the star indicates the central area of the ground where the survey was performed), and Bajo Oliveira with a map showing distribution and abundance of oysters estimated during the dredge survey (1987). Densities are number of oysters 10 m^{-2}.

one directed towards the assessment of the area covered by the oyster stock, the abundance distribution and the population structure; the second rendered additional information regarding population demography. The prospected area during 1987 had 122 km^2, with depths ranging from 4–22 m (Fig. 3). The survey was performed on a rectangular grid design, with legs parallel to the shore, each one divided in quadrats (1′ latitude by 1′ longitude). Stations were placed on the grid at regular intervals (one per quadrat) over each leg. The placement of sampling stations was made using radar and land references, and was corrected, when technically possible, by satellite readings. Each fishing tow lasted 10 minutes and swept an area of 2,700 m^2. Sixty stations were performed in four working days, recording initial and final depth, tow duration, and total unsorted catch weight. The catch from each haul was entirely stocked in 40 kg plastic bags. Three bags per haul (or the entire catch if not enough) were randomly sampled (or the entire capture in the case of poor hauls). The number of oysters, total weight of the oyster catch, and the weight of the rest of the components (inorganic and organic), were registered for each sampled bag. A sub-sample of each bag was used to size the oysters (TH in mm).

Over a total of six randomly chosen bags, separated on board, a detailed sampling was performed: oysters were sacrificed and "carriage" was assessed (number and size of epibiotic males on the platform of each oyster). Size (TH in mm) was recorded for each individual. Catch per haul data, obtained by multiplying the mean oyster catch in the sample by the total catch of the haul, were corrected by a gear efficiency factor of 15% (Iribarne et al. 1991). Catch and swept area per haul data were used to construct a map of density distribution on the ground. The area comprised between isopleths was estimated by computer.

Nine fishing hauls were performed at the N area of the ground during 1988 following the same procedure used in 1987. A sample consisting in one bag of clean catch (only oysters) was separated from the total catch of each haul. The oysters were sized and the epibiotic males on the platform of carrier oysters were counted and measured. The number of isolated and clustered oysters was reported for the entire catch of each haul.

The percentages of clustering and carriage between grounds were statistically compared by a Test of equality of percentages (Sokal & Rohlf 1969).

RESULTS

Spatial Distribution and Density

The Banco Reparo oyster ground occupies an area of 381,425 m^2 at depths ranging from 0.5–3 m at low tide. The ground lies in NE–SW direction, following the low tide mark, and parallel with the predominant tidal current (Fig. 2). Maximal density, 32 oysters 10 m^{-2}, was observed in a small area (0.24 km^2) located towards the SW side of the ground (Fig. 2, Table 1). It was not possible to identify a defined spatial pattern. Patches of variable density alternate, on the western side of the ground, over an area of shallow waters. The eastern side of the ground, on deeper waters, is a continuous fringe of low density. This limit is neatly marked and is defined by the presence of dense populations of Ophiuroids (Fig. 2).

At Las Grutas, oysters live on flat sandy areas as well as on limestone platforms and *Codium* prairies. However, the greatest concentrations were found on channels and depressions. The prospected area occupied a surface of, approximately, 2 km^2. Maximal density on the study area was 22 oysters per m^2. Mean estimated density for the complete prospected area was 8 oysters per m^2 (s = 5.3; N = 41).

TABLE 1.

Results of surveys carried out at Banco Reparo (1985) and Bajo Oliveira (1987). Description of strata (area and oyster abundance), mean oyster density and standard deviation per stratum.

Stratum Identifier	Abundance (Oysters·10 m^{-2}	Area (km^2)	Mean Density (Oysters·10 m^{-2})	SD
Banco Reparo				
I	0.1–0.5	0.77	0.23	0.15
II	0.6–2.4	1.21	1.27	0.51
III	2.5–5	0.95	3.58	0.69
IV	5.1–9	0.63	6.96	1.17
V	>9	0.24	12.9	3.09
Bajo Oliveira				
I	0.06–0.006	11.19	0.03	0.01
II	0.6–0.061	27.54	0.57	0.78
III	0.61–6	46.60	2.51	1.39
IV	>6	3.25	7.06	–

The oyster ground of Bajo Oliveira occupies an area of 88 km^2, forming a ground elongated from north to south within the isobats of 10 and 20 m (Fig. 3). The northern portion of the ground extends out from the 10 m isopleth, extending onshore to a depth of 4 m (Fig. 3). The higher density area (Fig. 3; Table 1) (3.25 km^2) was located in the NW portion of the ground. The mid density areas occupy a central fringe and represent a 52.60% of the total area. The western and eastern sides of the ground are defined by low-density patches (Fig. 3). The maximal oyster density reported at this ground was eight individuals per m^2. The population is concentrated in a narrow depth range (5–13 m). Fishing hauls were also concentrated in this depth range and mainly in the 10 m isobat (46.6%).

Population Structure

The population of Banco Reparo is homogeneous, lacking spatial segregation of adults and juveniles. Free oysters (non clustered) had a mean size of 68.69 mm (s = 17.34; N = 1327) (Fig. 4). All samples had a very low number of individuals smaller than 40 mm (<5%). The percentage of oysters forming clusters in the 1985 samples ranged from 0–34%. Over the total stock of oysters examined in 1985, 1986 and 1987, the percentage of clustering was 19% (N = 1574); 88% of the clusters composed of 2 oysters, 9% by 3 oysters, and 3% by 4 oysters (N = 114). No clusters comprising of more than four oysters were reported at this ground. Mean size of free oysters (χ = 77.93 mm; s = 13.3; N = 122) was only slightly higher than the mean size of clustered oysters (χ = 71.13 mm; s = 16.87; N = 122) (Fig. 4). The 1986 and 1987 samples enabled the completion of the demographic study of the ground including the component corresponding to the epibiotic oysters settled on the platform of carrier oysters. The size frequency distributions were clearly bimodal (Fig. 4). In 1986, the first mode (epibiotic males) was 10.69 mm (s = 7.06; N = 111), and the second (free oysters), was 70.62 mm (s = 15.09; N = 209). In 1987, the modes were 12.69 mm (s = 6.82; N = 152), and 77.56 mm (s = 12.15; N = 388).

The percentage of "carriage" (oysters carrying dwarf males), in oysters bigger than 50 mm, estimated from samples of the 1986 and 1987 surveys (N = 946), was 32%. The carrier oysters of

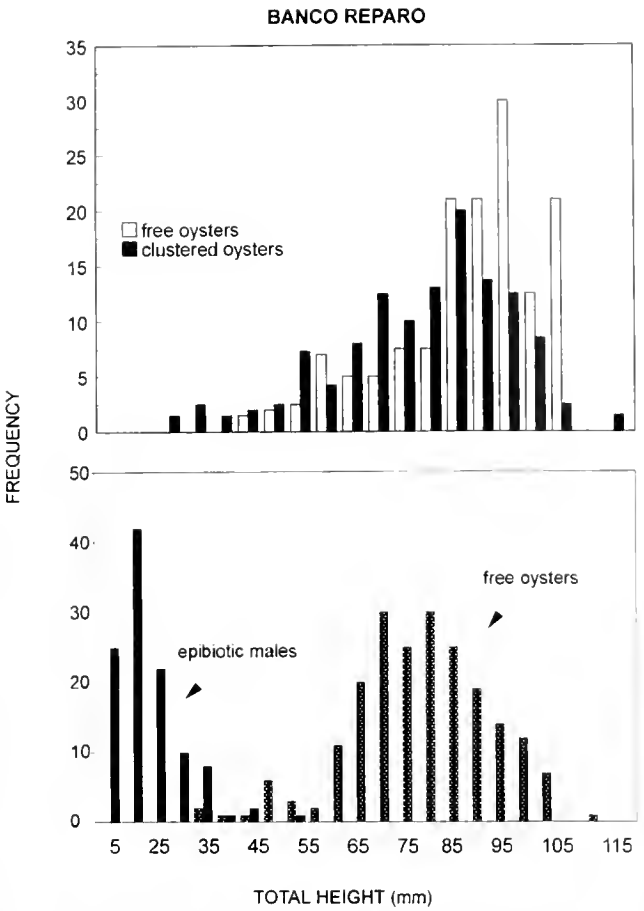

Figure 4. Banco Reparo. Size frequency distribution (total height in mm) of free and clustered oysters (above), and size frequency distribution of the complete population composed of free and epibiotic (dwarf males) oysters (below).

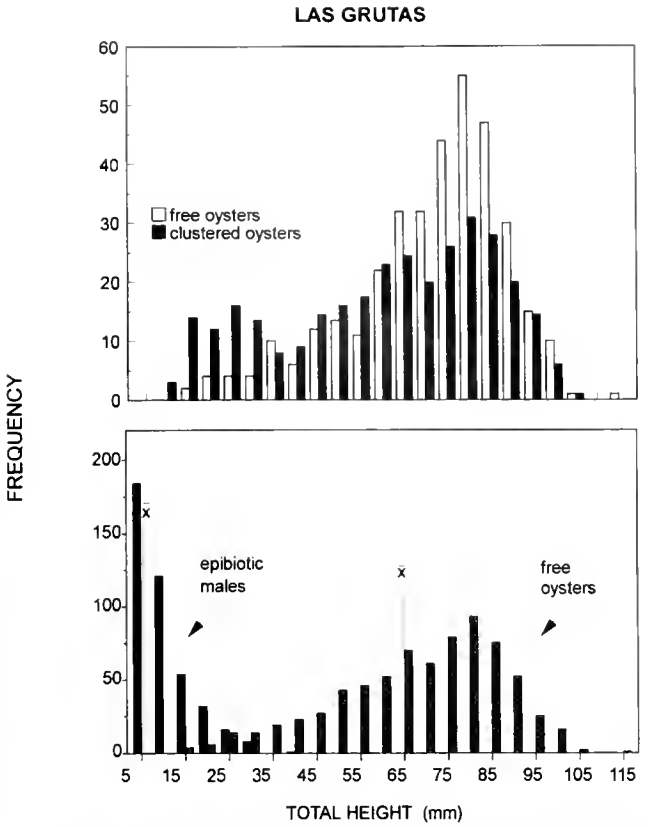

Figure 5. Las Grutas. Size frequency distribution (total height in mm) of free and clustered oysters (above), and size frequency distribution of the complete population composed of free and epibiotic (dwarf males) oysters (below).

Banco Reparo held a maximum of seven epibiotic males on the platform per oyster; 61% of the oysters carried one epibiotic male (mean = 1.69; s = 1.12; Table 2).

The total population of oysters at Las Grutas had a mean size of 66.67 mm (s = 18.67; N = 722). Free (non clustered) oysters had a mean size (χ = 69.05 mm; s = 18.18; N = 332) 10 mm higher than the group composed by clustered oysters (χ = 59.33 mm; s = 23.99; N = 317) (Fig. 5). The whole oyster population (including epibiotic males) presented a clear bimodal structure. The first mode, representing epibiotic males fixed on carrier oysters, was 8.2 mm (s = 6.3; N = 420), the second, representing the rest of the population, was 66 mm (s = 18.6; N = 722) (Fig. 5).

The clusters were composed of a maximum of 11 oysters of different sizes, those composed of only two oysters represented 47% of the total (mean = 3.26; s = 1.63). The proportion of clustered oysters was high in this ground: 44% of the sampled oysters (N = 627) had this life habit. The total estimated carriage (number of oysters >50 mm carrying epibiotic males) in the ground was 34% (N = 490). A maximum of six epibiotic males per carrier was registered, but 68% of the carriers (N = 259) were oysters carrying only one epibiotic male (Table 2).

The population of Bajo Oliveira is composed of large individuals. The mean size of oysters per haul in 1987 ranged from 77.6–94.2 mm (Fig. 6). Free oysters (isolated or clustered) presented a

TABLE 2.

Mean number of epibiotic males per carrier oyster at the three prospected grounds (Banco Reparo, Las Grutas and Bajo Oliveira).

Oyster Ground	N° of Epibiotic Males/Carrier Oyster	SD	N
Banco Reparo	1.69	1.12	365
Las Grutas	1.48	0.86	260
Bajo Oliveira	1.95	0.95	151

SD = standard deviation.

TABLE 3.

Percentages of clustering and carriage in oysters of the three grounds.

Ground	% Clustering	N	% Carriage	N
Banco Reparo	19	1574	32	946
Las Grutas	44	627	34	490
Bajo Oliveira	3.9	862	88.28	862
BR vs LG	ts = 3.68*		ts = 0.77 n.s.	
LG vs BO	ts = 20.31***		ts = 21.83***	
Br vs BO	ts = 11.64***		ts = 21.51***	

Banco Reparo (BR), Las Grutas (LG), and Bajo Oliveira (BO). Percentages (arcsin transformed) were compared between grounds by a test of equality of percentages (P = 0.001) (Sokal & Rohlf 1969).

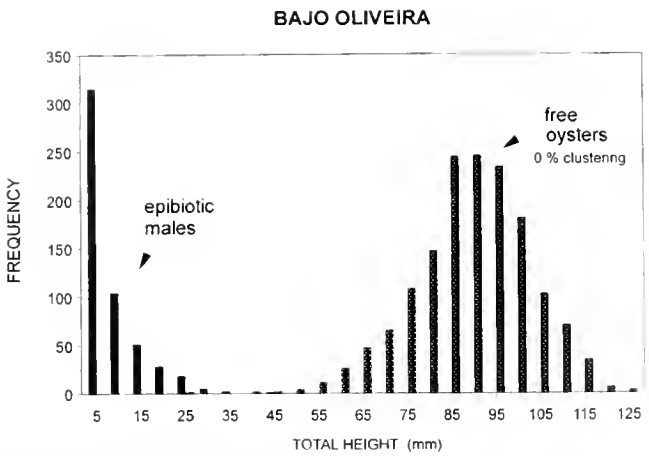

Figure 6. Bajo Oliveira. Complete size structure of the oyster population, including the component represented by dwarf males carried on the platform of carrier oysters. Samples collected on the 1987 survey.

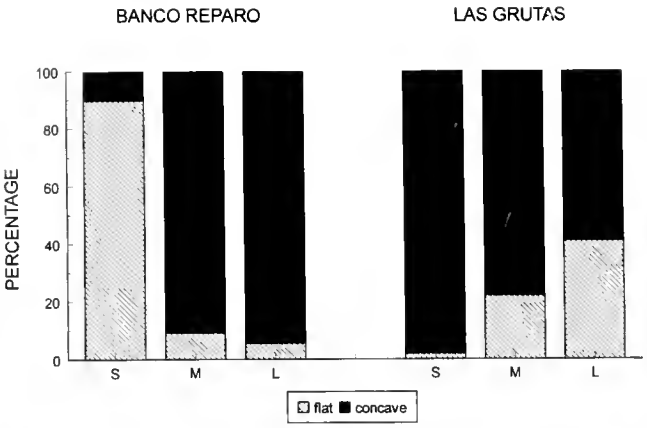

Figure 7. Life position of oysters at Banco Reparo and Las Grutas. Proportion of oysters living with its concave shell up ("concave"), and with its flat shell up ("flat"). Oysters were divided into three size groups: small (S = TH < 3 cm), medium (M = TH = 3–7 cm), and large (L = TH > 7 cm).

mean total height of 89.01 mm (s = 13.39; N = 1550) in 1987, and 90.2 mm (s = 7.52; N = 412) in 1988. The entire population, including epibiotic males, is bimodal (Fig. 6). The mode representing the epibiotic males is 9.09 mm (s = 6.53; N = 532). Carriage levels found on this ground were very high both in 1987 (83.6–97.6%) and in 1988 (74.19–100%). Mean carriage obtained pooling all samples was 88.28% (N = 862). Carrier oysters had a mean of 1.95 epibiotic males per oyster (s = 0.95; N = 151; Table 2) with five the maximum reported. The oysters of Bajo Oliveira are predominantly non-clustered. Pooling all data from 1987, 0% of clustering (N = 302) was found (Fig. 6), and 6.8% in 1988 (N = 560). Total clustering for both surveys was 3.9% (N = 862). All clusters were of two oysters.

The comparison of clustering and carriage percentages between grounds showed significant differences ($P < 0.001$) in all pairs of comparisons, with the exception of the percentages of carriage in Banco Reparo and Las Grutas where no significant differences were detected ($P > 0.001$) (Table 3).

Life position

A total amount of 1,858 oysters were sampled at Banco Reparo, 89.8% were found lying on their flat shell. Life position at this location appears related to the oyster's size with 81.8% of oysters smaller than 3 cm found lying on their concave shell and medium and large oysters (90.8% and 94.4%, respectively) found lying on their flat shell (Fig. 7).

Life position of 1,779 oysters was assessed at Las Grutas, 61% of which were lying on their flat shell. When data were separated by size groups, it was concluded that even when the proportion of oysters lying on their flat shell was higher, the percentages were almost equal in the case of large individuals (Fig. 7).

DISCUSSION

Surveys performed on the three main oyster grounds in the San Matías Gulf (Banco Reparo, Las Grutas and Bajo Oliveira) enabled us to conclude that the puelche oyster is distributed almost continuously along the northern coastal area of the San Matías Gulf, at depths ranging from 2–20 m.

The long planktonic larval life of this species (20 days, Pascual et al. 1989; Pascual and Zampatti 1995) suggests that larvae born in any of the studied grounds are capable of being transported long distances before settling. The grounds or high density areas, are likely interconnected by larval dispersion. This hypothesis rests upon the dynamics of residual coastal currents at the northern portion of the gulf (Lanfredi & Pousa 1988). The main current has two components: one flowing N–NE, and the second, flowing S–SE. The tidal wave follows the shoreline. Banco Reparo and Punta Villarino (Fig. 1) may then act as barriers, forcing the water to circulate describing a clockwise gyre.

The three studied grounds differed in their demographic characteristics. It was difficult to compare the grounds regarding their biomass due to the different assessment methods used. However, the results show that high oyster density areas were not large on any of the grounds, with maximal densities only on relatively small patches.

Oyster size structure at Banco Reparo and Las Grutas was similar. Size classes below 45 mm were better represented at Las Grutas than at Banco Reparo, even when on both grounds individuals <20 mm were not present in the samples. The Bajo Oliveira population showed a different demographic structure; it was represented by very large individuals, with mean size 20 mm larger than on the other two grounds, and no individuals <55 mm. Nowhere did we find patches of recruits, as is the case of other native gulf species such as the scallop, *Aequipecten tehuelchus*, or the mussel, *Mytilus edulis platensis*, suggesting that a clear spatial segregation of settlement does not occur in this species. On all three oyster grounds the portion of the population composing of individuals ranging from 1–30 mm was represented by epibiotic males.

The resulting scenario showed that, in the case of the puelche oyster populations, a distinction should be made between the **apparent demographic structure**, represented by adult and sub adult oysters, and the **real demographic structure**, that includes the small epibiotic oysters, an inconspicuous portion of the population. This "hidden" mode, added to the free portion of the population, shaped the bimodal size distributions typical of these populations. The apparent structure is intriguing in the case of a protan-

dric, sex alternating species, because the portion of the population that is absent, or poorly represented, corresponds to the free males (going through their first sexual maturation). These males are sexually more active than those in which the spermatogenesis occurs as a post-spawning event (females that become males after their first main spawning of the season) (Morriconi & Calvo 1979). In conclusion, the apparent population is primarily female.

The usual life position of free oysters is that in which the flat shell lies against the bottom. This orientation seems to be hydrodynamically more stable, facilitating water flow and minimizing the risk of being drifted by currents. The pattern observed at Banco Reparo, where this life position is exclusive for adult oysters, seems to be adequate for shallow and strong energy environments as an adaptation to resist smothering, condition under which the animals are capable of living for long periods.

Clusters are typical in this species, they are formed by the fixation and subsequent growing of recruits on a "founder" oyster (Morriconi & Calvo 1989). The incidence of this life habit differs among grounds (LG > BR > BO) (Table 3). At Bajo Oliveira, clusters are poorly represented (4%). This gradient among grounds is repeated by the number of oysters forming clusters (LG > BR > BO).

Morriconi and Calvo (1989) compared the incidence of this life habit in the three populations. The gradient that they described is similar to the one reported in this paper, even when the percentages of clustering are lower. This difference could be because their sampling lacked a specific design (Morriconi & Calvo 1989). The collection of samples in some spots of the ground may render a biased image of the overall structure. The authors describe, as well, the sex ratio of free and clustered oysters at Las Grutas, concluding that the proportion of males is higher on clustered (57.9%) than on free oysters (31.6%). This result would be expected if we analyze the size structure of both groups; clusters include a higher proportion of young oysters, ergo, of oysters still in their male protandric sexual phase. This result, however, is difficult to interpret because even when the authors mentioned that they dealt with individuals >50 mm, did not report the size structure of the studied oysters (Morriconi & Calvo 1989).

Here we propose that the different levels of clustering among grounds is a result of other regulatory mechanisms that operate on larval settlement occurring on living oysters, or on the survival of new settlers. These mechanisms, already proven, are mainly grazing (or bulldozing) by chitons, and smothering (Pascual 1997). Grazing accounts for 88% post-settlement mortality (Pascual 1997) of recruits settled on all hard surfaces, in the three months following recruitment. This mechanism, an ecological contingency, may explain the demographic differences among grounds where the mortality agents operate with varying intensity.

The proportion of carrier females is similar at Banco Reparo (32%) and Las Grutas (34%), but is considerably higher at Bajo Oliveira (88%) (Table 3). This topic was previously treated by Morriconi and Calvo (1989), who described the existence of a gradient in carriage, among grounds, inverse to the clustering gradient (LG < BR < BO). This result could again be the result of a biased sampling. The method we used for sample collection was of "total cleaning" of the sample area, thus avoiding bias in the representation of all the size classes present in the population. The similar percentages of carriage in Banco Reparo and Las Grutas are consistent with the similar size structure at both grounds.

The probability of "carrying" epibiotic males increases with the oyster's size. On oysters larger than 50–55 mm, time when carriage of epibiotic males begins, the platform develops, progressively widening. The growth of the epibiotic male produces, additionally, a hollowing on the platform that constitutes, once the epibiotic male detaches, an increasingly favorable substrate for new settlers (Pascual 2000). This development pattern of the platform with oyster size is expressed by the number of epibiotic males carried per oyster: at Banco Reparo and Las Grutas, 61% and 68% of the carriers respectively carry one epibiotic male, while at Bajo Oliveira, 63% of the carrier oysters hold two to five epibiotic males. The high percentage of carriage at Bajo Oliveira may then be explained by the larger size of the individuals that compose this population. The higher number of males per oyster at Bajo Oliveira suggests that recruitment could be heavier at this ground, even when the absence of oysters <55 mm in Bajo Oliveira suggests the opposite. Previous research focusing recruitment patterns on shells and platform of oysters suggest that mortality agents (mainly grazing or bulldozing) strongly operate on recruits at this ground during the three months following recruitment (Pascual 1997, Pascual 2000).

Considering different pieces of information regarding the three studied populations, and even when the age of the oysters cannot be determined in an absolute manner, we are able to conclude that the three populations differ in their dynamics, mainly in the turnover rates of individuals. At Bajo Oliveira, on one extreme, medium size individuals are absent and mean size of adult oysters is highest (89 mm). In comparison, at Las Grutas, intermediate sizes between the two modes are present and the mean size of adults is the lowest (66 mm). Banco Reparo occupies an intermediate position. Turnover rate is then maximal at Las Grutas, intermediate at Banco Reparo, and minimal at Bajo Oliveira.

The puelche oyster grounds at the San Matías Gulf offered the rare opportunity of studying the structure of one of the few natural and untouched flat oyster populations in the world.

The information gathered from the present work and from previous published information, enables us to draw the following scenario:

1. The puelche oyster grounds are mainly composed by low density populations.
2. Turnover rates in the three grounds are low even when Las Grutas shows a relatively higher rate.
3. Adults exert an attraction over the larvae ready to settle, live oysters being the main settlement substrate for oyster larvae in natural grounds (Pascual & Zampatti 1995; Pascual 2000).
4. Post-settlement mortality is very high (95–98%) mainly due to grazing by chitons (Pascual 1997).
5. Growth rate of individuals seems to be very low. The probability of carrying dwarf males on the shell platform increases as the size of the female oyster increases (Pascual 2000).
6. The male portion of the population is mainly represented by the epibiotic males fixed on the platform of female carrier oysters ("a hidden mode").
7. In natural grounds, 33 to 88% of the individuals >55 mm are "long living couples" composed by the female and its epibiotic dwarf male (Pascual et al. 1989).

The existing information provides enough reasons to protect this species, sustaining the fishing closure as a policy mainly based on the vulnerability of the stocks. Commercial exploitation of this species is likely to be supported by aquaculture.

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A COMPARATIVE STUDY OF ANTI-*PERKINSUS MARINUS* ACTIVITY IN BIVALVE SERA

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ABSTRACT The eastern oyster *Crassostrea virginica* has been decimated by a protistan parasite *Perkinsus marinus*; however, other bivalves appear to be more resistant to this pathogen. To better understand the basis for this difference in susceptibility, a comparative study of the activities of anti-*P. marinus* serum proteins of several bivalve species was carried out. Sera from mussels not known to develop *P. marinus* disease, *Mytilus edulis* and *Geukensia demissa*, contained high anti-*P. marinus* activity. About 25% of *M. edulis* serum samples contained <10 kDa anti-*P. marinus* peptides; the possibility of seasonal, geographic, or other reasons to explain this variability requires additional study. Anti-*P. marinus* peptides in *G. demissa* serum were apparently absent. Measurable anti-*P. marinus* activity was present in *C. virginica* and *C. gigas* sera, but at levels many hundred-fold lower than that of the mussels. The greater *P. marinus* resistance of *C. gigas* vs. *C. virginica* could not be explained by differences in anti-*P. marinus* activity of their sera. Hemocyte lysates from all the bivalves examined produced marked inhibition of the growth of *P. marinus*, suggesting that antimicrobial agents may be secreted by hemocytes into the serum. These factors may also participate in intracellular destruction of *P. marinus*, since the killing ability of the hemocytes of the different species closely mirrored the anti-*P. marinus* activities of their sera. The data suggest that *C. virginica* lacks active anti-*P. marinus* serum agents typical of *M. edulis* and *G. demissa*; however, *P. marinus* resistance of *C. gigas* seems not to depend upon elevated levels of antimicrobial serum factors.

KEY WORDS: *Perkinsus marinus, Crassostrea virginica, Crassostrea gigas, Geukensia demissa, Mytilus edulis*, antimicrobial proteins, defensins

INTRODUCTION

Circulating hemocytes form the basis for host defense in bivalve mollusks and many other invertebrates. Typically these cells mediate the destruction of foreign microorganisms encountered by chance contact in the hemolymph, or as a result of chemotactic migration (Cheng & Howland 1979). The microbes can be killed intracellularly or extracellularly by a variety of cytotoxic factors released from the cells. Many cytotoxic molecules have been described in the hemocytes and sera of bivalves such as lysosomal hydrolases (Pipe 1990) particularly lysozyme (McDade & Tripp 1967, Hardy et al. 1976), reactive oxygen species (Adema et al. 1991, Anderson 1996), reactive nitrogen species (Conte & Ottaviani 1995), as well as antimicrobial proteins and peptides (Roch et al. 1996). These activities may be constitutive, or may be stimulated or suppressed by various treatments. Furthermore, the expression of particular cytotoxic agents in bivalves can differ between species (Anderson 1994), seasons of the year (Santarém et al. 1994), and geographic locations (Fisher et al. 1996). Bivalves are generally able to mount an adequate defense against a majority of microbial invaders, thereby containing or resolving infections by evoking several complementary mechanisms of innate immunity. However, some pathogenic species, such as *Perkinsus marinus*, can subvert or escape these defense mechanisms in their susceptible bivalve hosts.

A number of small, cationic, cysteine-rich antimicrobial peptides have been described in bivalve mollusks, especially mussels. *Mytilus galloprovincialis* serum contains MGD1, a 4.4 kDa defensin-like peptide with 8 cysteines in its primary structure (Hubert et al. 1996a). Another isoform (MGD2) has recently been described, and both peptides are abundantly expressed in hemocyte granules (Mitta et al. 1999b). Bacterial challenge triggers release of MGD1 by the hemocytes into the cell-free hemolymph or serum (Mitta et al. 1999b). Peptides resembling arthropod defensins, with sequence similarities and 6 cysteines (defensins A and B) exist in the hemolymph of *Mytilus edulis* (Charlet et al. 1996). Another group of 4 kDa antimicrobial peptides have been found in mussels that are distinct from defensins; to date 5 isoforms are known: mytilins A and B from *M. edulis* (Charlet et al. 1996), and mytilins C, D and G1 from *M. galloprovincialis* (Mitta et al. 2000). Experimentally unchallenged *M. galloprovincialis* hemocytes and plasma contain other novel ~4 kDa antibacterial peptides (myticin A and B) containing 8 cysteines (Mitta et al. 1999a). In addition to these antibacterial molecules of mussels, an antifungal peptide of 6.5 kDa containing 12 cysteines was found in *M. edulis* serum (Charlet et al. 1996). Knowledge of antibacterial peptides of oysters and other bivalves is quite minimal. Antibacterial activity associated with small proteins or peptides was found in hemocytes and serum of *Ostrea edulis* and *Crassostrea gigas* (Hubert et al. 1996b).

The presence of lysozyme-like activity in bivalve hemolymph has been known for many years (McDade & Tripp 1967); it is another hemocyte-produced antimicrobial agent that can be released into the serum during bacterial challenge (Cheng et al. 1975). Lysozyme is thought to function in self-defense because it induces bacterial cell lysis by hydrolyzing β-1, 4 linked glycosidic bonds of cell wall peptidoglycan. Activities of bivalve lysozymes are often initially measured against *Micrococcus luteus*, although they are active against many other Gram-positive, as well as Gram-negative, bacteria. The approximate molecular mass of bivalve lysozymes range from 11 kDa (Nielsen et al. 1999) to 18 kDa (McHenry & Birckbeck 1979); they are thought to belong to a distinct family of invertebrate lysozymes (Jollès et al. 1996). Recently two molecules with lysozyme-like activity against *M. luteus* have been purified and characterized from bivalves: a 13 kDa protein with as many as 14 cysteine residues from *Tapes japonica* (Ito et al. 1999), and a 11 kDa enzyme (chlamysin) from the Icelandic scallop *Chlamys islandica* (Nilsen et al. 1999). The enzymes showed high protein structure stability and had high sequence homology to each other, but not to other known types of lysozyme.

A larger (320 kDa) cytotoxic polymeric protein has been described in *M. galloprovincialis* (Hubert et al. 1996b). It polymerizes to form a lytic complex that can disrupt the membranes of mammalian erythrocytes, mouse myeloma cells, and can kill

Bonamia ostreae, a protozoan parasite of the oyster *Ostrea edulis*. However, several bacterial species seem unaffected by this protein. This lytic activity in the plasma could be stimulated by sham injection or by injection of erythrocytes (Hubert et al. 1997).

The protistan parasite, *Perkinsus marinus*, is a major pathogen of the eastern oyster, *Crassostrea virginica*, causing mass mortalities in the Gulf of Mexico and the Chesapeake Bay. However, it is relatively nonpathogenic for other bivalves, including some species that share the same habitat with highly infected oysters. In order to better understand the basis for this marked difference in susceptibility, the results of comparative studies of anti-*P. marinus* serum proteins and peptides in several bivalve species are reported in this study.

MATERIALS AND METHODS

Bivalves

Local eastern oysters, *Crassostrea virginica*, were obtained from a commercial supplier; they originated from the Wicomico and/or St. Mary's Rivers, Maryland. Eastern oysters from Maine were purchased from the Pemaquid Oyster Company (Waldoboro, ME), and were collected in the Damariscotta River. *Crassostrea gigas* were purchased from the Taylor Shellfish Company (Shelton, WA), and had been collected from Totten Inlet, Puget Sound. Ribbed mussels, *Geukensia demissa*, were collected at Chincoteague, VA. *Mytilus edulis* were obtained at a local food store (their original source was Tenants Harbor, ME), collected by diving off Ocean City, MD, purchased from the Marine Biological Laboratory (Woods Hole, MA), or purchased directly from Great Eastern Mussel Farms (Tenants Harbor, ME).

The bivalves were held in aerated, recirculated water systems at 12 C, containing 25 ppt Instant Ocean (Aquarium Systems, Inc., Mentor, OH). Levels of nitrate/nitrite and ammonia were monitored and full or half water changes made whenever these parameters were outside the normal range. Bivalves were routinely acclimated in the tanks for at least one week before experimentation. The animals were fed live cultured algae or reconstituted frozen algal slurry 5 days per week. The live algae was a mixture of *Isochrysis galbana* and *Thalassiosira weissflogii*; the frozen slurry contained *Isochrysis* sp., *Chaetocerus gracilis*, and *Tetraselmis* sp.

Cultivation of Perkinsus marinus

Perkinsus marinus (strain I) cultures were originally provided by Drs. M. Faisal and J. La Peyre (Virginia Institute of Marine Science). The cultures were maintained in DME/HAM F-12 medium, with phenol red, reconstituted with 10 ppt artificial sea water and contained 1% penicillin-streptomycin solution, 2% fetal bovine serum, and was HEPES-buffered at pH 6.5. All the components of the medium were purchased from Sigma Chemical Co. This medium is a modification of that originally described by Gauthier and Vasta (1993).

*Anti-*Perkinsus marinus *Activity of Sera and <10 kDa Peptides*

Samples were withdrawn from the adductor muscle hemolymph sinus with a syringe equipped with a 1.5 inch 22 gauge needle. Samples were pooled from ≥6 bivalves and held at 1°C prior to centrifugation (300 *g*, 10°C, 10 min) to separate the hemocytes from the serum. The supernatant (serum) was filter-sterilized by passage through a 0.2 μm Whatman Puradisc syringe filter and frozen (−20°C) until further use, the cell pellet was discarded. Subsequently, the protein content of the serum was measured (BCA protein assay kit, Pierce Co.) and the desired concentrations reached by dilution with filter-sterilized Instant Ocean sea salts (Aquarium Systems, Inc.), adjusted to 25 ppt salinity (IO). Cultured *P. marinus* cells were washed and resuspended at a known density in DME/HAM F-12 media without phenol red, this *P. marinus* suspension was mixed with a dilution of serum to give a known final concentration of serum protein containing 1×10^5 *P. marinus* cells per ml, and incubated at 26°C. Sterile technique was used throughout all experiments. As indicated on the Figures, 0.5 ml aliquots were periodically removed from the culture flasks and read at 560 nm in a spectrophotometer; these OD readings were converted to numbers of *P. marinus* cells/ml from a previously constructed standard curve. In this way, the effects of various serum concentrations on the growth kinetics of this protistan were determined. This same basic method was also used to obtain a measurement of relative anti-*P. marinus* activity in the sera of the bivalve species under study. *P. marinus* cultures, set up as described, were in active growth phase for at least 200 h; exposure to serum during this period often markedly inhibited this growth. Therefore, the Ab_{560} of 170-hour cultures exposed to various serum concentrations were compared to the 170-h Ab_{560} of serum-free controls to determine the concentration of serum proteins required for 50% inhibition of normal growth (EC_{50}).

The anti-*P. marinus* activity of <10 kDa serum peptides was measured by the same turbidometric assay by substituting these peptides for the serum samples. About 9 ml of pooled, filter-sterilized bivalve serum was separated by ultrafiltration with Millipore Ultrafree Protein Concentrators by passage through 100 kDa and 10 kDa exclusion filters. Molecules passing through the 10 kDa ultrafilter were tested for anti-*P. marinus* activity. Preliminary data suggest that this activity may be lost after ≥1 month storage at −20°C.

Lysozyme Activity

Lysozyme activity was expressed as egg white lysozyme equivalents. Lysozyme dilutions (0–20 μg/ml) were prepared in 0.05 M HEPES buffer, pH 6.8. These standard solutions (0.25 ml) were mixed with 2.0 ml *Micrococcus lysodeikticus* (*M. luteus*) cell wall preparations (0.01 g/50 ml 0.05 M HEPES buffer) in order to produce a lysozyme standard curve. The ΔOD_{540}/min for each lysozyme concentration was measured spectrophotometrically. The resultant linear relationship between cell wall lysis and lysozyme concentration was used to quantify lysozyme levels in bivalve sera or <10 kDa peptides.

Inhibition of Perkinsus marinus *Cultures by Hemocyte Extracts*

Hemocytes were separated from hemolymph by centrifugation (300g, 10°C, 10 min), washed with IO, frozen at −20°C and thawed. Freeze-thawing was carried out three times, the lysate centrifuged (300g, 10°C, 10 min), and the cell debris pellet discarded. The supernatant was filtered (0.2 μm), analyzed for protein, permitting aliquots of known protein concentration to be added to *P. marinus* cultures; their effects on *P. marinus* growth at 170 hours were measured as described above.

*Anti-*P. marinus *Activity of Hemocytes*

This procedure was adapted from a tetrazolium dye assay used to assess the bactericidal activity of oyster hemocytes (Volety et al. 1999). Aliquots from 4–7d *P. marinus* cultures were centrifuged

and resuspended in ~0.4 original volume 25 ppt Instant Ocean containing 1% penicillin/streptomycin solution (IO/PS). The *P. marinus* density in this suspension was determined in a hemacytometer and adjusted to 1.2×10^7 cells/ml. Bivalve hemocytes were obtained and processed as previously described (Anderson et al. 1992), and a suspension of 2×10^6 hemocytes/ml was prepared. A 96-well microtiter plate was set up as follows: row one: 75 µl IO/PS, row two: 50 µl hemocytes and 25 µl IO/PS, row three: 50 µl hemocytes and 25 µl *P. marinus* (final ratio was 3 *P. marinus*/hemocyte), and row four: 50 µl IO/PS and 25 µl *P. marinus*. Different cell pools were used in each of 3 experiments, with 6 replicate wells for each experiment. The microtiter plate was incubated at room temperature (~21° C) for 2.5 h to permit cellular killing of the parasites. All wells then received 100 µl of DME/HAM F-12 medium without phenol red, and the plate was incubated (26° C, 17h) to provide a grow-out period for the surviving *P. marinus*. Then 20 µl MTS/PMS solution was added to all wells, following the directions for the Cell Titer 96 Aqueous Cell Proliferation Assay Kit (Promega Co.); the plate was vortexed and incubated an additional 2 h at 26° C. The absorbency of the wells was measured at 490 nm on a SpectraCount Plate Reader (Packard). The absorbance values of the reagent blanks (row 1) were subtracted from the readings of the wells in rows 2–4 to give corrected (corr) values and a killing index (KI) was calculated by the formula:

$$KI = 1 - \left(\frac{Abs\ row\ 3\ corr. - Abs\ row\ 2\ corr.}{Abs\ row\ 4\ corr.} \right) \times 100$$

RESULTS

Effect of Sera on Growth of Perkinsus marinus

At the seeding densities used in these studies, *P. marinus* cultures showed rapid growth from ~70–200 hr. The presence of *M. edulis* serum in the culture medium reduced *P. marinus* growth in a dose-dependent manner from 2–40 µg serum protein/ml DME-HAM medium (Fig. 1A). *G. demissa* serum was even more effective, with 20 µg /ml completely inhibiting growth for at least 200 hours (Fig. 1B). However, initial experiments using ≥600 µg/ml local *C. virginica* serum had an insignificant effect on *P. marinus* growth (data not presented).

*Comparison of Anti-*Perkinsus marinus *Activities*

The concentrations of serum proteins in *P. marinus* growth medium required to reduce the normal growth of the culture at 170 hr by 50% (EC_{50}) was determined for each bivalve. At 170 hr, normal growth was exponential and any serum-related inhibitory effects were evident (Fig. 1). Comparatively weak anti-*P. marinus* activity could be detected in the sera of *C. virginica* and *C. gigas* (Fig. 2). The EC_{50}s for locally collected *C. virginica*, Maine *C. virginica*, and *C. gigas* were ~2, 0.7, and 1.4 mg/ml, respectively. As was predicted from the preliminary observations (Fig. 1), the anti-*P. marinus* activity of mussel sera greatly exceeded that of oyster sera: *M. edulis* and *G. demissa* EC_{50}s were ~7 and 3 µg/ml, respectively (Fig. 3).

The <10 kDa serum peptides from local *C. virginica*, *M. edulis* and *G. demissa* were tested for anti-*P. marinus* activity. Such activity was never seen in peptides from *C. virginica* or *G. demissa*; however, about 25% of the *M. edulis* <10 kDa fractions tested showed comparatively strong anti-*P. marinus* activity (56.7 ± 11.2% growth inhibition). Lysozyme activity was also determined in serum and <10 kDa peptides from these bivalves. Lysozyme levels in *C. virginica* serum were high (20.3 ± 2.3 µg/ml) as comparted to those in *M. edulis* (1.4 ± 0.7) and *G. demissa* (3.9 ± 0.6). Some lysozyme-like activity was recorded for the <10 kDa fraction from *C. virginica*, but only trace levels were seen in the peptides of *M. edulis* (0.02 ± 0.08) or *G. demissa* (0.11 ± 0.13).

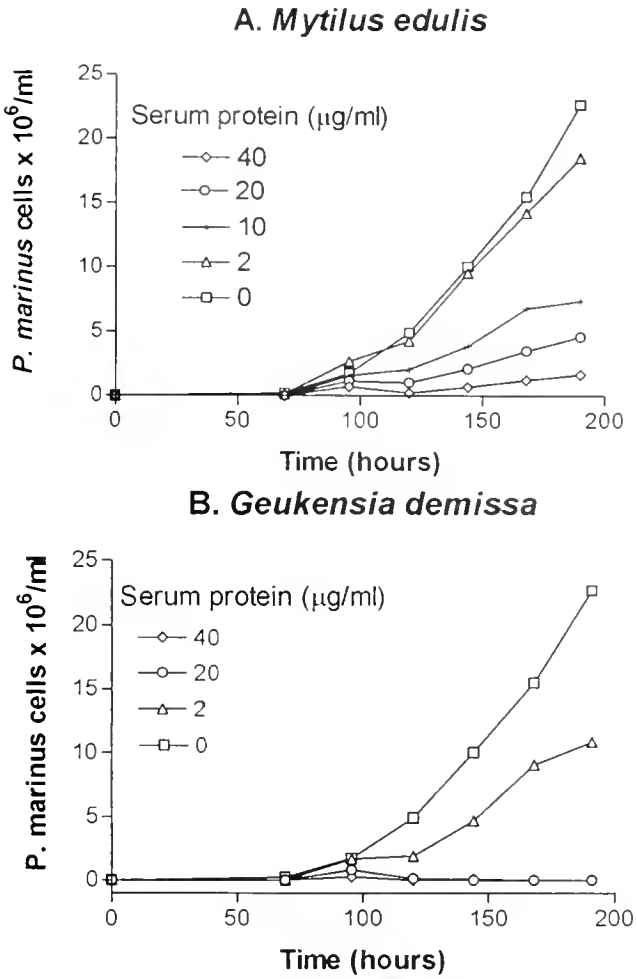

Figure 1. The effect of the presence of mussel sera on the growth of *P. marinus*. Growth kinetics were inhibited by ≤40 µg/ml serum proteins from *M. edulis* (A.) and *G. demissa* (B.)

Inhibition of Perkinsus marinus *Growth by Hemocyte Extracts*

Anti-*P. marinus* activity was also present in the hemocytes of the bivalves in this study (Fig. 4). Protein extracts from these cells markedly inhibited the growth of *P. marinus* cultures, especially lysates from *M. edulis* and *G. demissa*. The activity of *C. virginica* (local) hemocytes was somewhat lower, but apparently greater than that of the serum, on a per µg protein basis.

Hemocyte-mediated Killing of Perkinsus marinus

Laboratory-propagated *P. marinus* was actively killed *in vitro* by hemocytes of the mussels (*G. demissa* and *M. edulis*); however, both local and Maine *C. virginica* and *C. gigas* hemocytes showed much lower and more variable killing ability (Fig. 5). Anti-*P.*

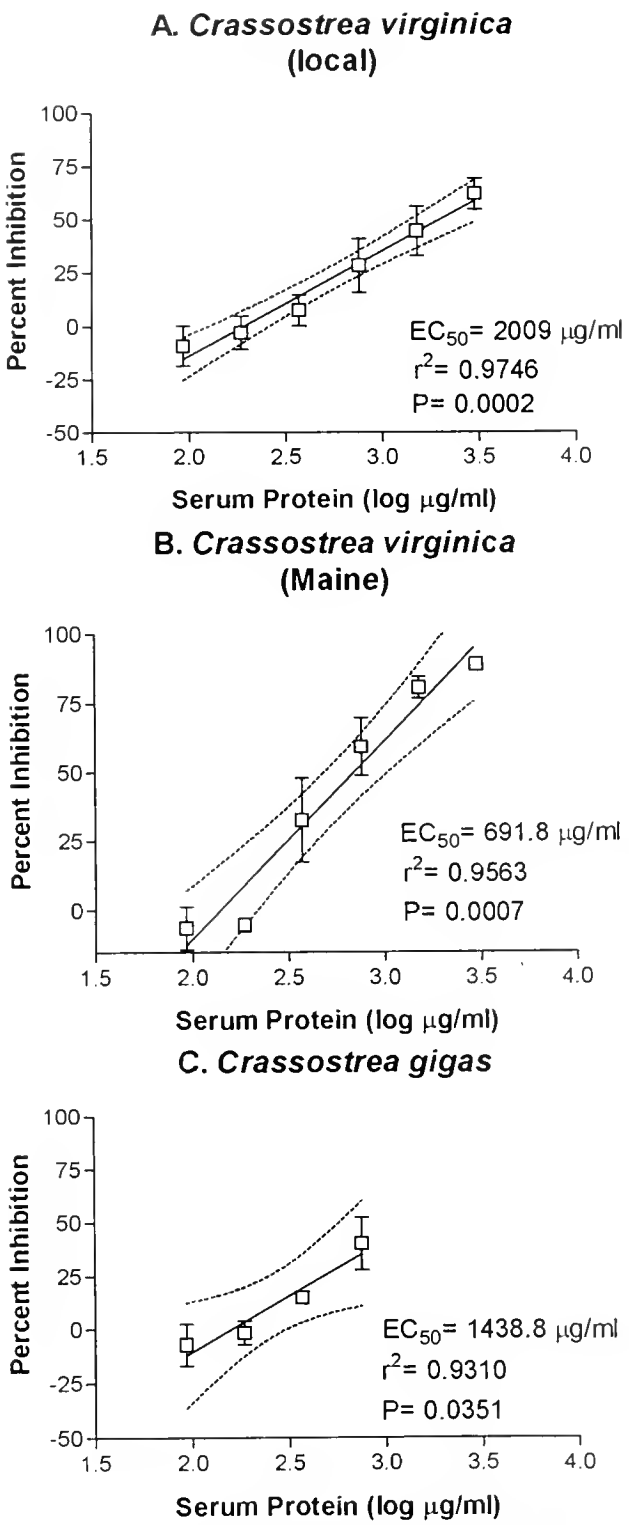

Figure 2. Dose-dependent inhibition of *P. marinus* by oyster sera: Maryland *C. virginica* (A.), Maine *C. virginica* (B.), and *C. gigas* (C.). The EC_{50} values represent the mean µg oyster serum protein/ml medium (n = 5) required to reduce normal *P. marinus* growth at 170 hr by 50%.

marinus capacity of hemocytes from the mussel species was significantly greater than that of the oysters ($P < 0.05$, Students *t* test). The cellular pattern of activity mirrored that of the hemocyte extracts.

DISCUSSION

In order to learn more about the basis for the differential resistance to the *C. virginica* pathogen *P. marinus* shown by other bivalves, a comparative study of cytotoxic serum proteins and peptides was carried out. The species selected include local *C. virginica* with light-moderate *P. marinus* infections, uninfected Maine *C. virginica* that were minimally exposed to *P. marinus in situ*, *C. gigas* that are reported to be more resistant to *P. marinus* disease than *C. virginica* (Meyers et al. 1991), and two mussels *M. edulis* and *G. demissa* that may be naturally exposed to *P. marinus*, but develop few pathological consequences. Progression of any *P. marinus* infections in oysters in these studies was retarded by maintenance at 12°C. In this study, the bivalves were not treated in any way to experimentally modulate the activity of naturally-occurring anti-*P. marinus* serum factors. However, it is possible that collecting, laboratory maintenance, infection of oysters by *P. marinus*, and/or infection of the bivalves by other microorganisms could have influenced the levels present in the sera. In light of the reported inducibility of many antimicrobial substances, experiments are planned to quantify the effects of *P. marinus* infection and other stressors on the expression of cytotoxic proteins and/or peptides.

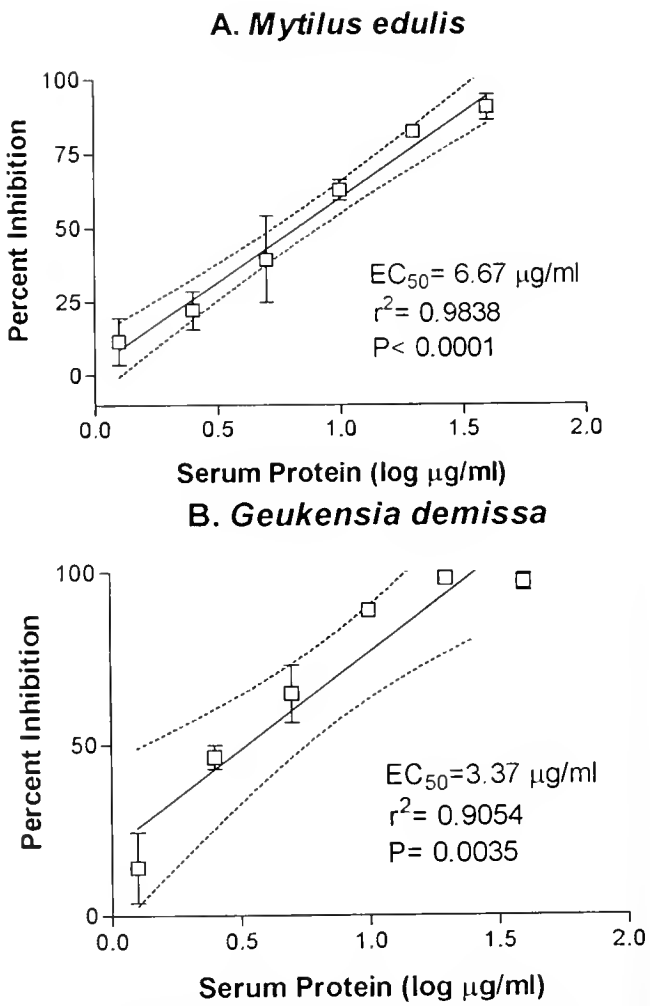

Figure 3. Dose-dependent inhibition of 170 hr *P. marinus* cultures by serum proteins from *M. edulis* (A.) and *G. demissa* (B.). The EC_{50} values are calculated as described for Figure 2.

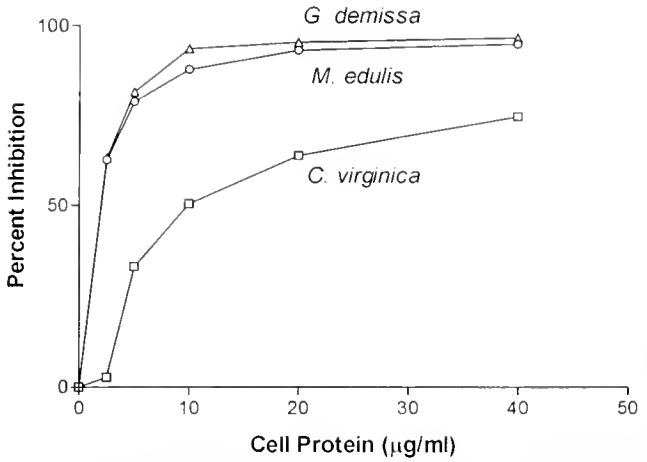

Figure 4. Inhibition of 170 hr *P. marinus* cultures by hemocyte lysates from *G. demissa*, *M. edulis*, and local *C. virginica*; hemocyte lysates from Maine *C. virginica* and *C. gigas* were not analyzed.

The mussel sera had many hundred-fold greater anti-*P. marinus* activity than *C. virginica* sera; this might be expected because of their apparently greater resistance to the parasite, since they are rarely infected. Antimicrobial molecules in *M. edulis* and *M. galloprovincialis* are well known (see Introduction), but this is the first report of such activity in *G. demissa*. As little as ~7 μg/ml *M. edulis* serum protein could inhibit *P. marinus* growth by 50%, *G. demissa* serum was about twice as active. By comparison, about 2 mg/ml local *C. virginica* serum was required to produce comparable inhibition of *P. marinus*. It is interesting that anti-*P. marinus* activity of *C. gigas* serum was slightly, but not statistically significantly, more active than local *C. virginica*. The possible role of serum factors in *C. gigas* resistance to *P. marinus* has not been clarified. The apparently higher anti-*P. marinus* activity of sera from Maine oysters versus local oysters is also hard to explain. In Maine, oysters are rarely infected by *P. marinus* probably because of its geographic location near the Northern range of the parasite. The lower anti-*P. marinus* activity in local oysters may be a consequence of, or a contributing factor to, their *P. marinus* infections, or a reflection of strain differences in oysters collected from distant sites.

The <10 kDa fractions of the sera are of interest because in these would be found defensins or other antimicrobial peptides. There was no evidence of anti-*P. marinus* activity in this fraction of *C. virginica* or *G. demissa* serum, but high activity was occasionally seen in the <10 kDa *M. edulis* fraction. This implies that different molecules are involved in the strong anti-*P. marinus* activities of the two mussels. The activity of *Mytilus* spp. antimicrobial peptides against protistan parasites has received comparatively little study. The inhibitory activity of *M. edulis* serum was first noted by Gauthier (1998). MGD-1, a defensin from *M. galloprovincialis* was found to be inactive against *P. marinus* (Hubert et al., 1996a), but the other molluscan peptides have not been assayed for anti-protistan activity. However, antimicrobial peptides from other animals have shown this activity: megainin 1 (from the skin of *Xenopus laevis*) killed *Bonamia ostreae*, the intrahemocytic parasite of the oyster *Ostrea edulis* (Morvan et al. 1994) and polyphemusin (from hemocytes of *Limulus polyphemus*) inhibited growth of *Perkinsus marinus* at 8 μg/ml (Pierce et al. 1997). Both of these authors have considered the possibility of using genes coding for antimicrobial peptides to produce disease-resistant, transgenic mollusks; this possibility has also been discussed by Roch (1999).

In *C. virginica* serum lysozyme levels were comparatively high (~20 μg/ml), but its putative role in anti-*P. marinus* defense has yet to be definitively established. Chu and La Peyre (1989) reported low levels of lysozyme activity in Chesapeake Bay oysters in the summer, when levels of *P. marinus* infection are high, but Fisher et al. (1996) found the opposite for oysters from Florida. Reports on lysozyme levels as influenced by *P. marinus* infection in field-collected oysters are conflicting, but experimental infection has little effect on levels of the lysozyme in *C. virginica* or *C. gigas* (La Peyre et al. 1995). While there is little doubt that lysozyme in bivalve sera plays a protective role by virtue of its antibacterial activity, there are several lines of evidence to diminish the likelihood that it has much effect against *P. marinus*. *C. gigas* is more resistant to *P. marinus* than *C. virginica*; however, lysozyme levels in *C. virginica* are much higher than *C. gigas* (La Peyre et al. 1995). The same conclusion can be drawn from the data in this study, where the anti-*P. marinus* activities of *M. edulis* and *G. demissa* sera are many hundred-fold that of *C. virginica*, but the lysozyme levels are quite low in the mussels as compared to the oyster. It appears that the agent(s) in *M. edulis* and *G. demissa* serum responsible for anti-*P. marinus* activity have yet to be identified. The data above suggests that lysozyme may not be important. Likewise, antimicrobial peptides may not contribute much to the anti-*P. marinus* activity of mussel sera. No anti-*P. marinus* activity was ever detected in the <10 kDa fraction of *G. demissa* serum and such activity was only occasionally seen in *M. edulis* peptides, but all unseparated mussel serum samples had very high anti-*P. marinus* activity, regardless of the presence or absence of active peptides.

In addition to serum components such as antimicrobial peptides, lysosomal hydrolases, and recognition factors, the 320 kDa cytotoxic polymeric protein described by Hubert et al. (1996b) in *M. galloprovincialis* serum will be looked for in future studies. This seems like a particularly promising candidate for an anti-*P. marinus* protein in light of its activity against *Bonamia ostreae*.

When hemocytes from the oysters and mussels in this study were lysed, comparatively high levels of anti-*P. marinus* activity could be measured (Fig. 4). The activities of *M. edulis* and *G. demissa* hemocyte extracts were higher than that of *C. virginica* (local). These data suggest that the agents responsible for anti-*P.*

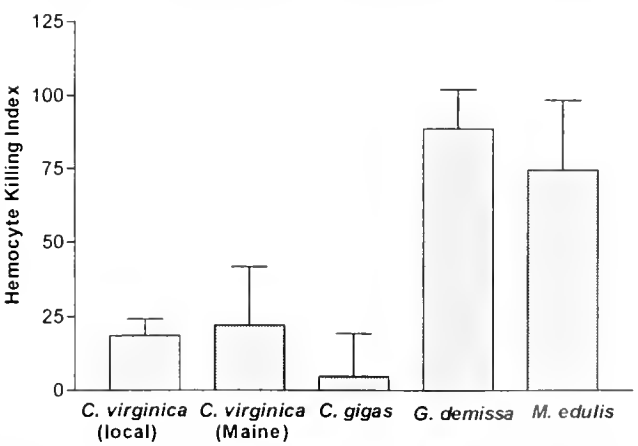

Figure 5. The *in vitro* ability of oyster and mussel hemocytes to kill *P. marinus*. Height of the bars indicate mean % *P. marinus* killed in 2.5 hr ± SD, n = 4.

marinus activity in the sera probably are synthesized by and released from the hemocytes, as is the general case for bivalves (Cheng 1992, Mitta et al. 1999b). The mussels >oysters pattern of anti-*P. marinus* activity seen in the sera and cell extracts was also recorded when the ability of hemocytes to kill *P. marinus* in vitro was tested (Fig. 5). *C. virginica* hemocytes were able to kill ~25% of the parasites and *C. gigas* hemocytes killed ~10%, but there was considerable variation and no significant differences between the oyster hemocyte killing indices. Since *C. virginica* has very limited ability to destroy *P. marinus* in vivo, it was surprising that a modest hemocyte killing index was consistently recorded. A similar finding was reported by Volety and Fisher (2000), in which hemocytes from Floridian oysters averaged 57% *P. marinus* killing capacity. Since it is likely that *P. marinus* loses some of its virulence during laboratory culture (Bushek et al. 1997); it would be interesting to test the ability of hemocytes to kill *P. marinus* freshly harvested from naturally infected oysters. In any case, it appears that resistance of *C. gigas* to *P. marinus* cannot be explained by enhanced capacity for hemocytic killing. In contrast to the oysters, *G. demissa* and *M. edulis* cells routinely and efficiently destroyed *P. marinus in vitro*.

In conclusion, the data suggest that low-level anti-*P. marinus* activity can be detected in the serum and cells of *C. virginica* and *C. gigas*, but differences between these species are insignificant and cannot be used to explain reported differences in resistance to *P. marinus* disease. However, the serum and cells of the mussels *G. demissa* and *M. edulis* have high levels of anti-*P. marinus* activity. In all bivalve species tested, the hemocytes contained anti-*P. marinus* activity, and it is likely that the hemocytes secrete serum antimicrobial agents. It appears that the high anti-*P. marinus* activity characteristic of *M. edulis* and *G. demissa* sera may depend on cytotoxic molecules other than, or in addition to, lysozyme or antimicrobial peptides.

ACKNOWLEDGMENTS

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HISTOPATHOLOGY OF THE INFECTION BY *PERKINSUS ATLANTICUS* IN THREE CLAM SPECIES (*RUDITAPES DECUSSATUS*, *R. PHILIPPINARUM* AND *R. PULLASTRA*) FROM GALICIA (NW SPAIN)

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ABSTRACT In this study, we have determined the histopathology of the infection by the protozoan parasite *Perkinsus atlanticus* in three exploitable clam species (carpet-shell clam *Ruditapes decussatus*, Manila clam *R. philippinarum*, and the species *R. pullastra*) from Galicia (NW of Spain). In histological preparations from infected animals, typical mature trophozoites of *Perkinsus*, containing lipid droplets and vacuole but no vacuoplast, were observed. The trophozoites were usually in groups surrounded by a light halo. Pre-palintomic tomonts, as well as palintomic tomonts with a variable number of daughter cells, were also present in the host tissues. Less frequently, immature trophozoites with an undifferentiated cytoplasm were observed. The presence of *P. atlanticus* cells was always associated with a strong hemocytic infiltration of the surrounding tissues. Occasionally, the parasite cells were internalized by granular hemocytes. The morphology and distribution of the *P. atlanticus* were similar in the three clam species studied, this being the first time that *R. pullastra* from Galicia has been found infected by *P. atlanticus*. The morphology of the parasite's life cycle stages and the histopathology of the infection resemble those reported on other species of the genus *Perkinsus*.

KEY WORDS: *Perkinsus atlanticus*, protistan, Protozoa, *Ruditapes decussatus*, *Ruditapes philippinarum*, *Ruditapes pullastra*, histopathology

INTRODUCTION

Protozoan parasites of the genus *Perkinsus* (Apicomplexa, Perkinsea) have been reported from marine molluscs all around the world. *P. marinus* (Mackin et al. 1950) has been associated with heavy oyster mortalities in the East and Gulf coasts of the USA since it was first described (Andrews 1996, Burreson et al. 1994). *P. olseni* parasitizes the abalone (*Haliotis ruber*) in Australia (Lester & Davis 1981), and the recently described *P. qugwadi* infects the Japanese scallop (*Patinopecten yessoensis*) in Canada (Blackbourn et al. 1998). Until now, the only *Perkinsus* species described in Europe has been *P. atlanticus*, parasite of the carpet-shell clam (*Ruditapes decussatus*) in Portugal (Azevedo 1989). On the other hand, *Perkinsus* sp. infections have been described in Manila clam (*Ruditapes philippinarum*) from Japan (Hamaguchi et al. 1998, Maeno et al. 1999) and Korea (Choi & Park 1997), in the pearl oyster (*Pinctada maxima*) and 30 other molluscan species from Australia (Goggin & Lester 1987, Norton et al. 1993), and in more than 30 marine bivalve species in different areas of the Pacific and Atlantic oceans (Perkins 1988).

In Spain, *Perkinsus* sp. has been found in the carpet-shell clam (*Ruditapes decussatus*) and the Manila clam (*R. philippinarum*) from the South Atlantic, Mediterranean and Cantabric coasts (Cigarría et al. 1997, Montes et al. 1995a, Navas et al. 1992, Sagristà et al. 1995). Two other bivalve molluscs of economical importance, *R. pullastra* and *Venerupis aureus*, have been reported to be infected by this parasite in Huelva (SW Spain) (Navas et al. 1992).

In Galicia (NW Spain), clam aquaculture is an activity of high economical importance. The production of *Ruditapes decussatus*, *R. pullastra* and *R. philippinarum* in this region in 1998 totaled 914, 2554 and 604 Tm respectively, this representing an annual income of approximately 4900 million pesetas (1 US$ = 185 pesetas) (data from the Consellería de Marisqueo, Pesca y Acuicultura of the Xunta de Galicia). The presence of a *Perkinsus*-like organism was associated with severe carpet-shell clam mortalities in a depuration plant in Meira (Galicia) thirteen years ago (Figueras et al. 1992, González Herrero et al. 1987). Later, *Perkinsus* sp. was detected in carpet-shell clams and Manila clams from different locations along the Galician coast (Figueras et al. 1996). A strong hemocytic infiltration was always observed in the *Perkinsus*-infected tissues, this probably affecting the clam health and endangering its production (Figueras et al. 1992). Recently, Novoa et al. (2001), using molecular tools based on the 18S rRNA gene, have demonstrated the identification of the *Perkinsus* species observed in carpet- shell clams from Galicia as *P. atlanticus*.

This study was conducted to describe the histopathology of the *Perkinsus atlanticus* infection in the three economically most important clam species in Galicia: carpet-shell clam, Manila clam and *R. pullastra*.

MATERIALS AND METHODS

Clams

Market-sized carpet-shell clams (*Ruditapes decussatus*), Manila clams (*R. philippinarum*) and R. *pullastra*, were collected in Campelo (Ría of Pontevedra), Meira, Vilaboa and Arcade (all Ría of Vigo), and Camariñas (Central Ría) (all Galicia, NW Spain) (Fig. 1). Approximately, one hundred individuals were examined from each location. The animals were processed immediately after being received in the laboratory.

Diagnosis of Perkinsus

The presence of *Perkinsus atlanticus* infection in the clams was assessed using Ray's Fluid Thioglycollate Medium (RFTM). Briefly, one pair of gills from each clam was introduced into Fluid Thioglycollate Medium (FTM) (Ray 1954, Ray 1966), and incubated at room temperature in the dark for three to five days. Then,

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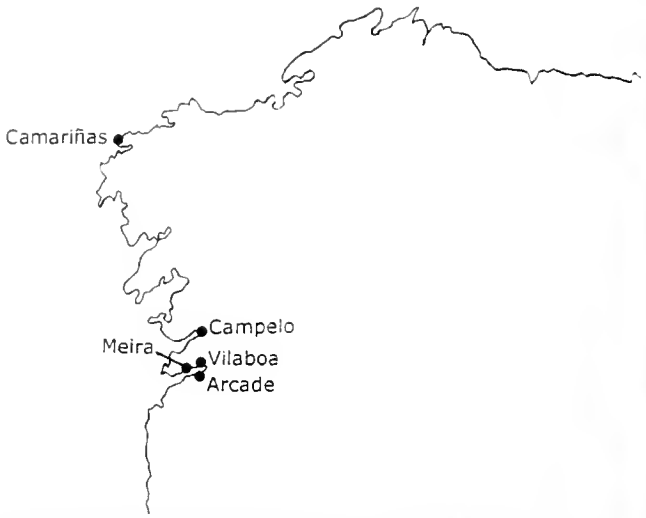

Figure 1. Clam sampling sites in the Ría of Pontevedra (Campelo), Ría of Vigo (Meira, Vilaboa and Arcade) and Central Ría (Camariñas), all of them located in Galicia (NW Spain).

gills were placed on a slide, stained with Lugol's iodine and observed with a light microscope.

Histology

The remaining soft tissues were fixed in Davidson's fixative (Shaw & Battle 1957) for 24 hours and a transverse section approximately 5 mm thick including mantle, gonad, digestive gland, gills, kidney, and foot, was excised from each clam. Tissue samples were embedded in paraffin and 5-µm sections were stained with hematoxylin-eosin. The histological sections were observed under a light microscope (Nikon).

RESULTS

The RFTM-treated gills of several carpet-shell clams, Manila clams and *R. pullastra* showed dark blue spherical bodies, typical of hypnospores of *Perkinsus*. Only those clams diagnosed as positive for *Perkinsus* by RFTM were examined histologically.

Macroscopically, the most heavily infected individuals showed milky-white cysts in their body surface. Under the microscope, the morphology and distribution of *Perkinsus atlanticus* life cycle stages in the host tissues were similar in the three clam species studied, with strong hemocytic infiltration of the infected tissues and encapsulation of the parasite (Fig. 2, Fig. 3, Fig. 4). The histopathology of the infection by *P. atlanticus* in *Ruditapes decussatus* is described in detail below.

The parasite cells were mainly located in the gills and connective tissue close to the digestive epithelium and digestive tubules (Fig. 4A). In heavy infections, the parasite was detected in all clam organs examined, including the gonads and the muscle tissues (Fig. 4B). Granular hemocytes containing one (Fig. 5A) to ten (Fig. 5B) *P. atlanticus* cells were frequently observed.

The most prevalent *P. atlanticus* life cycle stage present in the clam tissues was the mature trophozoite (Fig. 6). Its large eccentric vacuole lacked a vacuoplast, and a dark nucleolus was present inside the nucleus (Fig. 6C). The trophozoites were usually grouped in clumps of 2 to 20 cells that were usually similar in size

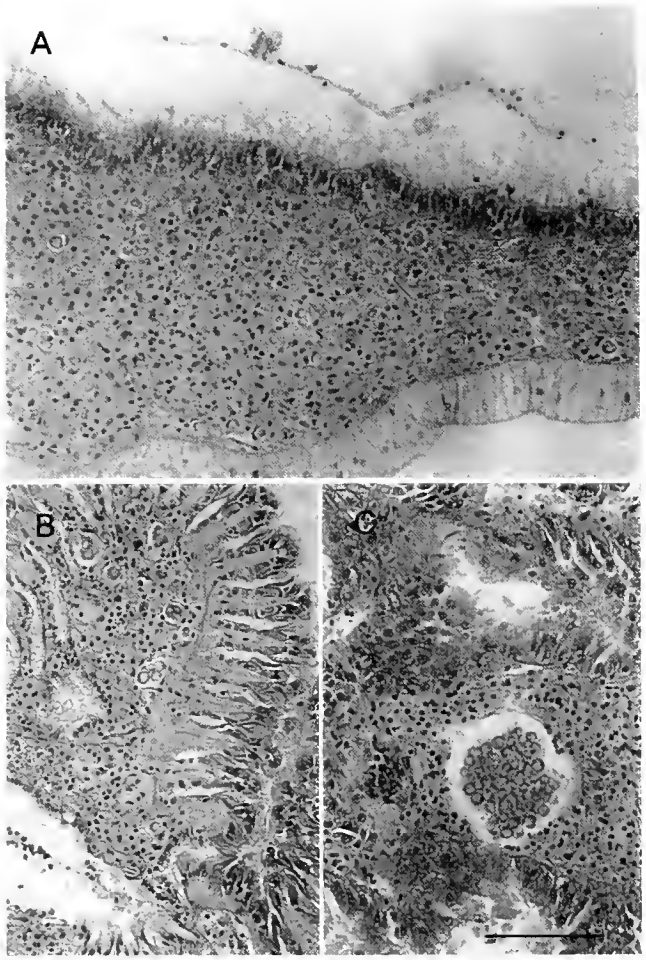

Figure 2. Mature trophozoites of *Perkinsus atlanticus* in Manila clam (*Ruditapes philippinarum*) tissues. This stage has a large eccentric vacuole without a vacuoplast, and a dark nucleus. (A) *Perkinsus atlanticus* located in the mantle edge. Note the strong hemocytic reaction associated with the infection. (B) Clam's gills heavily infected by *Perkinsus atlanticus*. In this case, the parasite cells are grouped in small clumps (fewer than five individuals per clump). Most of the groups are surrounded by a light halo. (C) Accumulation of *Perkinsus atlanticus* trophozoites in the tissue surrounding digestive tubules. The halo around the large parasite clump is surrounded by numerous hemocytes. Bar = 100 µm.

and stage. Palintomic cells, named tomonts by Perkins (1996), were also frequently detected in the clam tissues (Fig. 7). They were larger than mature trophozoites, and were not usually found clustered. Before the onset of palintomy, mature trophozoites lost the vacuole, which mixed with the cytoplasm (Fig. 7A). This prepalintomic stage was similar in size to the tomont, and showed a dense undifferentiated aspect.

Once the palintomy started, two- and four-daughter-cell tomonts were clearly identified in the clam tissues (Fig. 7B, Fig. 7C, Fig. 7D). When the number of daughter cells was too high, the tomont had a granulated aspect with several dark nuclei (Fig. 7E). Sometimes, the vacuole did not disappear from mature trophozoites before the palintomy and during palintomy underwent divisions to give the daughter cells inside the tomont (Fig. 7B, Fig. 7C, Fig. 7F). Occasionally, small immature trophozoites without vacuole were observed. Their cytoplasm was undifferentiated but nucleus and nucleolus were well defined (Fig. 5B, Fig. 7G, white

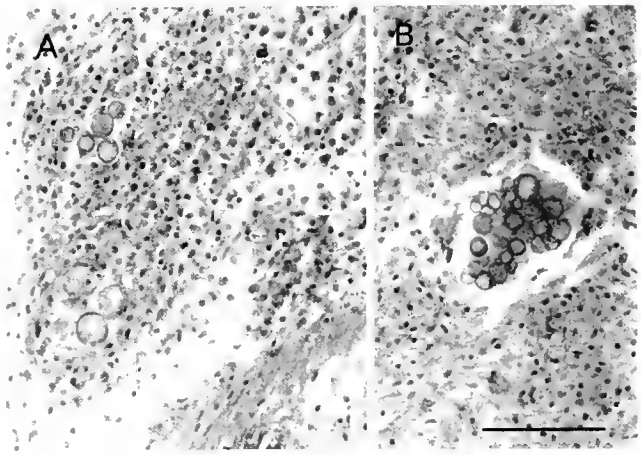

Figure 3. Mature trophozoites of *Perkinsus atlanticus* in *Ruditapes pullastra* tissues. Note the similarity of these parasite cells with those present in Manila and carpet-shell clam tissues. The trophozoites have a large eccentric vacuole and a dark nucleus, and are associated with strong hemocyte infiltration. They can appear individually (A) or in large clumps (B). In the latter case, a light halo usually surrounds the grouped parasite cells. Bar = 50 µm.

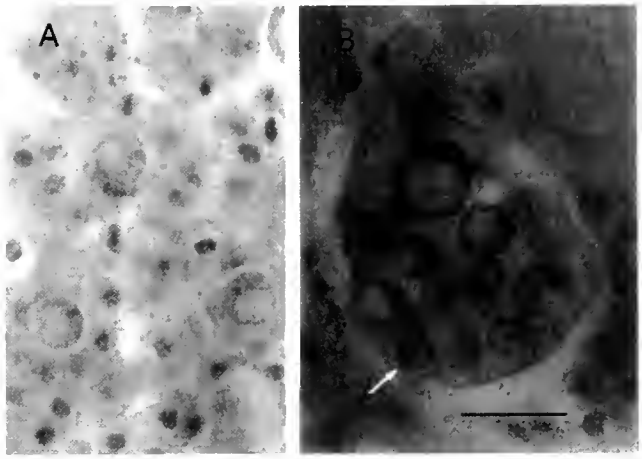

Figure 5. Mature *Perkinsus atlanticus* trophozoites internalized by carpet-shell clam (*Ruditapes decussatus*) hemocytes, probably granulocytes. (A) Hemocytes containing one *P. atlanticus* cells and (B) containing to ten *P. atlanticus* cells. Bar = 25 µm.

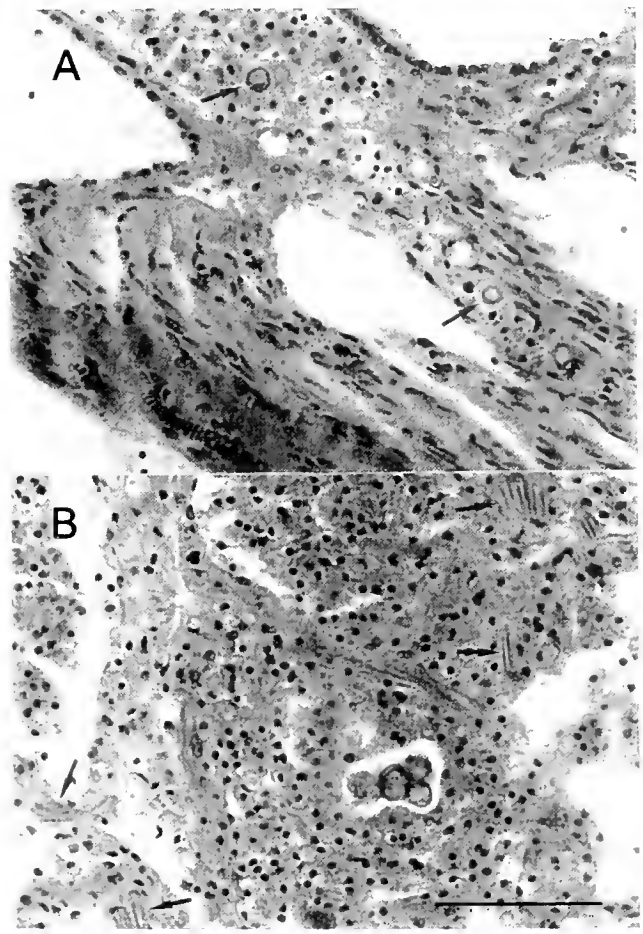

Figure 4. Mature trophozoites of *Perkinsus atlanticus* in carpet-shell clam (*Ruditapes decussatus*) tissues. The morphology of this life-cycle stage, with a large eccentric vacuole and a dark nucleus, is similar to that of the *Perkinsus atlanticus* present in Manila clams and *R. pullastra*. (A) *Perkinsus atlanticus* (arrows) in the gill epithelium. Note the strong hemocytic reaction and the disorganization of the tissues. (B) *Perkinsus atlanticus* in the muscle tissue. The clump, constituted by four mature trophozoites, is surrounded by a light halo. Among the numerous hemocytes, pieces of muscle tissue are seen (arrows). Bar = 50 µm.

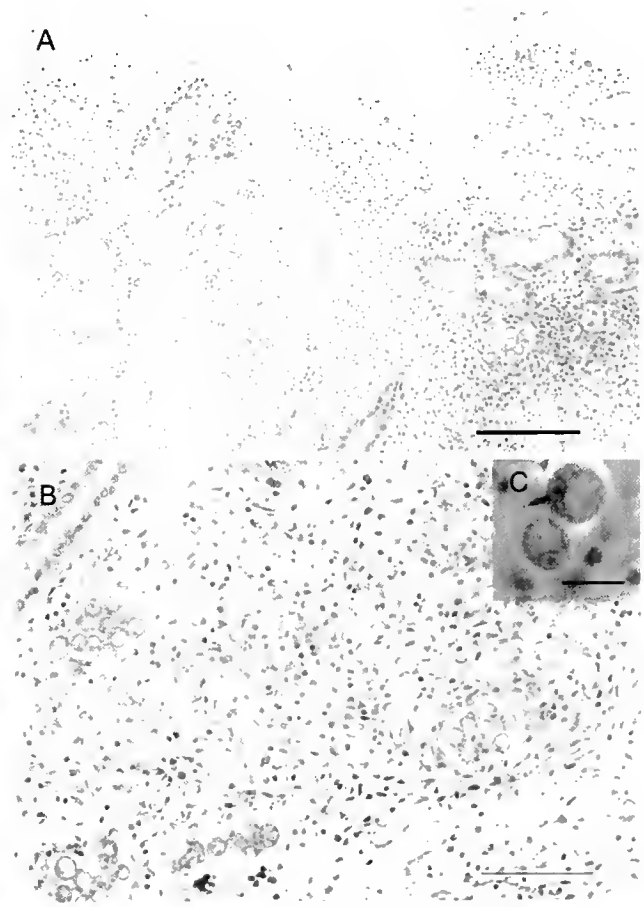

Figure 6. General view of carpet-shell clam (*Ruditapes decussatus*) tissues heavily infected with *Perkinsus atlanticus*. Note the strong hemocytic infiltration (A) and the light halos surrounding the parasite clumps (B). The most prevalent stage is the mature trophozoite (detail in C), with an eccentric vacuole and a nucleus containing a dark nucleolus. Immature trophozoites are also present in the clam tissue (B), with an undifferentiated cytoplasm and no vacuole. Several dark lipid droplets are present in their cytoplasm. The immature trophozoites are grouped and surrounded by a light halo. Bar = 100 µm (A), 50 µm (B), and 15 µm (C).

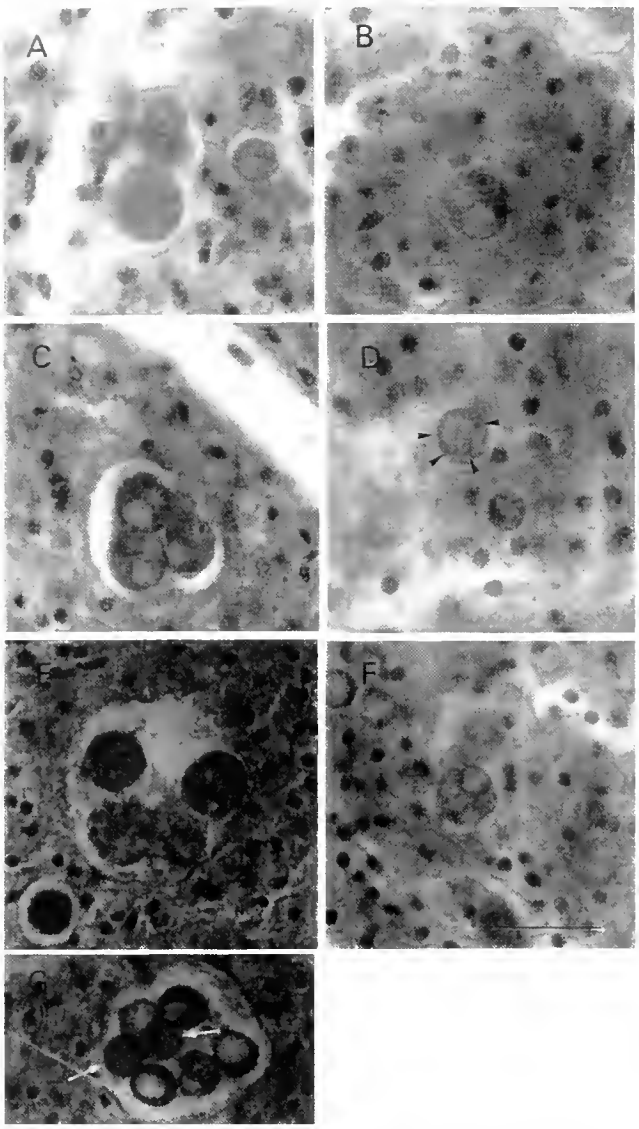

Figure 7. Palintomy of *Perkinsus atlanticus* in the carpet-shell clam (*Ruditapes decussatus*) tissues. (A) Mature trophozoites after loosing the vacuole by its mixing with the cytoplasm. The nucleus is distinguishable as a dark spot. Two- (B, C) and four- (D) cell daughter tomonts. Nuclei of the four- cell tomont are pointed with arrowheads. (E) Tomont in advanced palintomy, with a high number of daughter cells whose nuclei are observed as dark spots. (B, C, F) Palintomy of trophozoites whose vacuoles were not lost prior to palintomy. The vacuole is successively divided with the cytokinesis (B, C), until the tomont acquires a multivacuolated aspect (F). (G) Small immature trophozoites without vacuole, with undifferentiated cytoplasm but nucleus and nucleolus well defined (white arrows). Bar = 25 μm.

arrows). Generally, the diameter of the protozoan cells infecting the host tissues varied from 3–15 μm.

Frequently, a light halo, which resembled an empty capsule, surrounded the clumps of parasite cells (Fig. 2C, Fig. 4B, Fig. 6). A high number of hemocytes were grouped around these capsules. The presence of every life cycle stage of *Perkinsus atlanticus* was associated with a strong host hemocytic reaction (Fig. 2, Fig. 3, Fig. 4, Fig. 6). When the infection was heavy and the *P. atlanticus* cells were distributed in all host tissues, they looked disorganized with massive hemocyte infiltration.

DISCUSSION

In general, the morphology and distribution of *Perkinsus atlanticus* in the tissues of the three clam species from Galicia were similar to those reported before for *Perkinsus* infecting different host species around the world (Lester & Davis 1981, Azevedo 1989, Montes et al. 1995a, Sagristà et al. 1995, Perkins 1996, Blackbourn et al. 1998, Hamaguchi et al. 1998, McLaughlin & Faisal 1998a, Bower et al. 1999, Maeno et al 1999). Cultured *P. marinus* and *Perkinsus* sp. cells have been reported to develop vacuolated immature trophozoites inside the tomont (Perkins 1996, McLaughlin & Faisal 1998b), as we found for *P. atlanticus* in the clam's tissues. On the other hand, and in contrast to *P. marinus* (Perkins 1996), no vacuoplast was observed inside the *P. atlanticus* vacuole. *P. olseni* also lacks this inclusion (Lester & Davis 1981). The presence of a *Perkinsus* species in *R. pullastra* has only been reported in the South Spanish Atlantic coast (Navas et al. 1992). Therefore, this is the first time that *Perkinsus* is described in such economically valuable clam species in Galicia.

Recently, a new protozoan parasite morphologically similar to *Perkinsus* has been described in carpet shell clam from Galicia (Figueras et al. 2000). Moreover, this new species, named *Pseudoperkinsus tapetis*, develops hypnospores in RFTM indistinguishable from *Perkinsus* hypnospores. In spite of the coexistence and visible similarity of the two organisms (Ordás & Figueras 1998, Novoa et al. 2001), the parasitic cells observed and described in this paper should be mainly *Perkinsus* since its prevalence is much higher than that of *P. tapetis* (Novoa et al. 2001).

The initial response of the host to the *P. marinus* infection is the hemocytosis and the migration of hemocytes to the infection site (Lauckner 1983). This could be the origin of the strong hemocytic infiltration associated with the presence of *P. atlanticus* in the clam tissues. When the *P. atlanticus* infection rate is very high, the accumulation of hemocytes can be macroscopically observed as white cysts as previously reported by Lauckner (1983), Azevedo (1989), and Sagristá et al. (1995).

The encapsulation of the parasite is associated with the secretion of a "specific" protein by the granulocytes that had migrated to the infection place and surround the parasite cells (Montes et al. 1995b, Montes et al. 1996, Montes et al. 1997). In this study, the capsules that surrounded the *P. atlanticus* clumps seemed empty. This could be due to the histological processing that might have extracted the capsule material. However, *P. marinus* releases proteases when cultured in vitro (La Peyre et al. 1995) that alter several host defense parametres (Garreis et al. 1996) and degrade certain proteins of the oyster hemolymph (Oliver et al. 1999). Therefore, it could also be possible that *P. atlanticus* secretes similar proteases in vivo, these being responsible for the digestion of the capsule material and interference with the host defense response.

The oyster tissues with higher prevalence of *Perkinsus marinus* are the gills and the digestive gland (Lauckner 1983, Oliver et al. 1998). The hypothesized strategy of *P. marinus* for entering into the host (through feeding) would explain this distribution (Lauckner 1983, Andrews 1988). In the carpet-shell clam, the connective tissue of the organs shows the highest *Perkinsus* infection degree (Chagot et al. 1987). These results agree with our observations of the clam tissues infected with *P. atlanticus*.

Cheng (1988) defined *Perkinsus marinus* as an extracellular parasite, although it can be phagocytized by the hemocytes and be destroyed intracellularly. In contrast, Perkins (1996) considers

that, although the parasite cells can be found free in the host tissues, they are most commonly located in the phagosomes of the hemocytes. In any case, we have frequently observed one to ten *P. atlanticus* cells inside a clam hemocyte. If the hemocyte is not able to destroy the internalized *Perkinsus* cells, the parasite could grow inside the blood cell until finally destroying it, thus diminishing the host cellular defence factors. However, a relationship between the hemocyte lysis and the capsule formation around the *Perkinsus* cells has been reported by Montes et al. (1995b, 1996, 1997), constituting a host reaction to the hemocyte destruction by the parasite proliferation. The morphology of the host hemocytes that have internalized the *P. atlanticus* cells corresponded to granular hemocytes. In fact, it is well known the higher phagocytic activity of the granulocytes compared to the hyalinocytes in bivalve molluscs (Fisher 1986, Feng 1988).

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EFFECTS OF *PERKINSUS MARINUS* ON REPRODUCTION AND CONDITION OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*, DEPEND ON TIMING*

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ABSTRACT The protistan parasite *Perkinsus marinus* has been responsible for high mortality of eastern oysters, *Crassostrea virginica*, along the Atlantic and Gulf Coast of the United States. It also causes sublethal effects, although an impact on reproduction has heretofore been difficult to demonstrate. We examined the effect of *P. marinus* infection on growth, reproduction (as measured by relative gonadal size, and the proportional representation of gametogenic tissue and gametes in the gonad), and condition index of eastern oysters. Oysters of known age and genetic history were exposed to *P. marinus* infection and sampled at 2–4 week intervals over an 11-month period during the 1990–1991 epizootic in Delaware Bay. During this time, 50 to 100% of the oysters had detectable infections and nearly 55% died. Shell growth rates were inhibited as infections intensified during initial exposure in autumn 1990, but resumed in the spring and remained high, even after infection became heavy. The effect on reproduction varied with the stage of the gametogenic cycle. The relative gonadal size and the proportion of gametogenic tissue in the gonad, as well as the condition index, were most strongly depressed during the spring gametogenic period; however, there was no significant effect of parasite burden when the oysters were reproductively mature and ready to spawn. Data suggested that infected oysters, recovered, produced gametes, and spawned in early summer. Other studies have found inconsistent evidence of sublethal deleterious effects of *P. marinus* on reproduction. By sampling throughout the year and employing oysters of known genetic background, of the same age, reared in a common environment, and for which the timing of initial infection was known, we were able to show that *P. marinus* does have significant negative effects on oyster reproduction, measurable primarily during gametogenesis, rather than at spawning.

KEY WORDS: *Perkinsus marinus*, oysters, reproductive stage, reproduction, parasite effects, condition index

INTRODUCTION

In Delaware Bay, two major protozoan parasites, *Perkinsus marinus* (Mackin, Owen, Collier 1950, Levine 1978) and *Haplosporidium nelsoni* (Haskin, Stauber, Mackin 1966) infect oysters, *Crassostrea virginica* (Gmelin 1791) (Ford & Haskin 1982, Ford 1996). These parasites cause severe physiological disruption in the host oysters, often leading to death (Ray et al. 1953, Ford & Figueras 1988, Choi et al. 1989, Ford & Tripp 1996, Paynter 1996). They have been recognized as a cause of oyster mortality along the east coast of the United States for at least 40 years (Ford & Tripp 1996). While *H. nelsoni* has been prevalent in Delaware Bay since the late 1950s, *P. marinus* did not become epizootic in until the late summer of 1990 (Ford 1996). Before that, only sporadic and very low levels of this parasite were found in the Bay. The sudden upsurge of *P. marinus* infections is probably due to a history of introductions of infected oysters into Delaware Bay and an environmental warming trend that allowed the parasite to become epizootic (Ford 1996, Cook et al. 1998).

Decreased reproduction is one common effect of parasites on their hosts (Thompson 1983, Barber et al. 1988, Forbes 1993, Choi et al. 1994, Ford & Tripp 1996). While infection by *H. nelsoni* has been shown to have a significant sublethal effect on reproduction (Barber et al. 1988), similar evidence for *P. marinus* has not been definitive (Choi et al. 1994, Kennedy et al. 1995, Paynter 1996). Kennedy et al. (1995) were able to detect some deleterious effects of *P. marinus* infection on gametogenic characteristics, but not in a consistently predictable pattern. Barber (1996) found that *C. virginica* exposed to infections by both *H. nelsoni* and *P. marinus* in Virginia showed little gametogenesis at all; however, the relationship between level of parasitism and gonadal stage in individual oysters was not reported. To explain variable effects of infection, there has been speculation that oysters can divert energy from growth to reproduction when infected with *P. marinus* (Choi et al. 1994, Kennedy et al. 1995, and Hofmann et al. 1995). Both reproduction and energy storage in bivalves have distinct seasonal cycles. *Perkinsus marinus* infection and subsequent disease development also have seasonal patterns influenced by environmental temperature and salinity (Andrews 1988, Ford & Tripp 1996, Kennedy 1996). To properly assess the effects of parasites on reproduction and condition of their hosts under these conditions, it is necessary to sample at all stages of both the infection and reproductive cycles. In this study we measured the effects of *P. marinus* on the eastern oyster by examining individuals of known genetic background and age, reared in a common environment. We followed the effects of *P. marinus* from the first signs of disease in early August of 1990 until the majority of the oysters had heavy infections at the end of June of 1991. Our goal was to quantify the effect of parasitism on the oysters at different stages of the reproductive cycle. Oysters are a commercially important species and a significant component of the estuarine ecosystem. Therefore, a direct quantification of the impact of the parasite on host reproduction, and the timing of this impact, will provide useful information for management decisions (Powell et al. 1994, Kennedy 1996, Krantz & Jordan 1996, Powell et al. 1997).

MATERIAL AND METHODS

Test Oysters

Strains of *C. virginica* have been maintained in a common environment (lower Delaware Bay) for multiple generations since the 1960s, as part of a selective breeding project for resistance to

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*This paper is dedicated to F. James Rohlf on the occasion of his 65th birthday.

MSX disease, caused by *H. nelsoni* (Haskin & Ford 1979, Ford & Haskin 1987). In 1988, oysters from Long Island Sound origin (Rutgers LA strain) and Delaware Bay origin oysters (Rutgers XF strain) were spawned to produce pure lines and reciprocal crosses. These 1988 year-class animals were seventh generation representatives of the inbred MSX disease-resistant strains produced in the Haskin Shellfish Research Laboratory breeding program. All were produced and reared under identical conditions (see Dittman et al. 1998).

Sampling

We determined reproductive state, condition index, and parasite infection intensities by sampling forty oysters at 2-week (spring, summer, and fall) to 4-week (winter) intervals from August 1990 to July 1991. Equal numbers (N = 10) of offspring from each pure line and their reciprocal crosses were sampled. Similar size distributions (as measured by maximum shell dimension) were sampled on each collection date to minimize the effect of absolute size on the estimates of reproductive measurements and condition index (Galtsoff 1964, Rainer & Mann 1992). Shell sizes of 400 haphazardly chosen individuals were measured every three weeks to monitor shell growth. Dead oysters were counted and removed at regular intervals and cumulative mortality was computed over the study period.

Sample Processing

The shell height (longest dimension from umbo to growing edge) of each oyster was measured with a calipers to the nearest 1 mm. Whole live weight, (wet shell and soft body mass) was measured to the nearest 0.1 g. The shells were opened and the body was removed. Soft body mass was measured to the nearest 0.1 g. Air-dried valves were weighed to the nearest 0.1 g. The rectum was removed and cultured for *P. marinus* in Ray's fluid thioglycollate medium (RFTM, Ray 1966). Oysters were individually tagged and fixed in Davidson's solution for 24 hours, and then placed in 70% ethanol for storage until histological analysis was conducted.

To eliminate possible confounding effects of MSX disease on the analysis, all oysters were examined for *H. nelsoni* infections by standard tissue section histology. The oysters were processed using the procedure of Barber et al. (1988) for combined condition index measurement and histology. In this procedure, the whole fixed tissue was weighed and a standard 5-mm-transverse section through the gills and visceral mass between the palps and the adductor muscle was removed. The section and the remaining tissues were then weighed separately. The remaining tissue was dried for the calculation of individual condition index. The section was dehydrated through an alcohol series, cleared in xylene, embedded in paraffin, and sectioned at 5 μm. Tissue sections were stained with iron hematoxylin, acid fuchsin, and aniline blue. The slides were used for the measurement of reproductive condition, as well as to determine the presence and intensity of *H. nelsoni*.

Reproductive Measures

The Gonadal Index (GI), which is the proportion of the cross-sectional visceral mass area occupied by the gonad, is a useful estimate of fecundity in oysters provided individuals with similar environmental histories are compared (Barber et al. 1988, Heffernan & Walker 1989, Morales-Alamo & Mann 1989). In our study, the GI was estimated by image analysis. In addition, the proportion of the gonad occupied by all gametogenic tissue (gamete producing follicle tissue and gametes) and by gametes alone was estimated using point-count stereology on two randomly chosen (×400) microscope fields of each oyster (Elias et al. 1971).

Condition Index

The Condition Index (CI) is a standard measure used in shellfish growth and reproductive-cycle analyses (Widdows 1985). It is an indication of long-term changes in individual nutrient status and is a useful measure of the metabolic state of bivalves, including that produced by stress (Rainer & Mann 1992). It is often used to demonstrate seasonal physiological changes in natural populations (Lawrence & Scott 1982). We determined CI as the proportion of the internal shell volume occupied by dried soft tissue:

$$CI = (\text{dry body mass (g)} / \text{internal shell volume (mm}^3)) \times 100.$$

Internal shell volume was estimated as the difference between whole live weight and valve weight (Lawrence & Scott 1982). Total dry body mass was adjusted to account for the section removed for histology by assuming that the wet mass of the section had the same proportion of water as the rest of the tissue.

Infection by Parasites

Rectal tissues incubated in RFTM were stained with Lugol's Iodine, spread on a slide, and the level of P. *marinus* infection was rated on the Ray/Mackin scale of 0–5 using a compound microscope (Ray 1954, Mackin 1962). A 0 rating indicated that no were parasites detected, while a rating of 5 indicated that the oyster tissues were almost completely replaced by parasites. There is an exponential relationship between the Ray/Mackin scale and parasite abundance in the tissue (Choi et al. 1989), so when single or few parasites were found in the tissue samples, we gave a rating of 0.5 rather than 1 to allow us to distinguish, for the purpose of testing for parasite effects, between a very low level of detected infection and the relatively higher rating of 1. For the sake of simplicity, we will term oysters in category 0 "uninfected", although we recognize that they have a high probability of being parasitized (Bushek et al. 1994). Sample infection levels are reported as prevalence and the weighted prevalence (WP), which is the mean of all infection intensities including those rated 0.

Tissue slides of all oysters were examined for the presence and intensity of *H. nelsoni* (Ford & Haskin 1982). Oysters with advanced or systemic *H. nelsoni* infections were eliminated from analyses so as not to confound measurement of the effect of *P. marinus* (Ford & Figueras 1988, Ford et al. 1990).

Statistical Analysis

To facilitate analysis, we grouped the oyster samples into six periods according to published descriptions of the seasonal reproductive cycle (Loosanoff 1942, Ford & Figueras 1988, Thompson et al. 1996). The reproductive patterns of the oysters in this study fit the published descriptions. The first period was August and September of 1990, the end of spawning for that summer, when the animals were reabsorbing gametes. The second period was October to mid-December, when the oysters were reproductively inactive. The third period encompassed early gametogenesis, from January to early April 1991. The fourth period was from mid-April through early May, when the oysters were undergoing rapid gametogenesis. The fifth period was mid-May to early June, when the oysters were fully ripe and beginning to spawn. The last period

was from the middle to the end of June 1991, when the oysters were in late spawning.

When the samples within infection classes were <5, they were pooled for the analysis. In addition to the oysters eliminated from analysis because of *H. nelsoni* infections, two animals with trematode, *Buccephalus* sp., infections were excluded, because this parasite often castrates its hosts (Ford & Tripp 1996). A total of 679 oysters were analyzed.

Two-way ANOVAs were performed to test the effects of sampling date and parasite burdens on reproductive measures and condition index (Sokal & Rohlf 1981). Preliminary tests indicated no significant effect of strain so that all 4 groups were pooled for further analysis. A separate ANOVA was performed for each of the six reproductive periods. Multiple comparisons among infection-category means during each reproductive period were made using the Tukey-Kramer method (Sokal & Rohlf 1981, Gagnon et al. 1990). Correlation analysis was performed to determine the relationship between shell size and *P. marinus* intensity during infection peak in the autumn of 1990 and the early spring of 1991. Differences were considered significant at $p = 0.05$.

RESULTS

Perkinsus marinus infections were first detected in the experimental oysters in mid August of 1990, at which time prevalence was 18%. All but one infection were category 0.5; one oyster had a level 1 infection. Prevalence and intensity increased through fall and early winter, then declined gradually over the winter and spring, reaching a low in early May 1991 (Fig. 1, Table 1). Both measures then increased dramatically from mid May to early June 1991. Mortality rose to 30% between the beginning of August and early November 1990, ceased over the winter and early spring, and resumed in mid May 1991 (Fig. 2). Between mid May and the end of June, when the final sample was collected, 30% of the remaining oysters died, raising the total cumulative mortality to about 55%. The high death rate continued after the study ended so that by September 1991, an estimated 97% of the oysters had died.

Despite the high disease pressure, the mean size of oysters increased over the study period (Fig. 3). A lag in the rate of

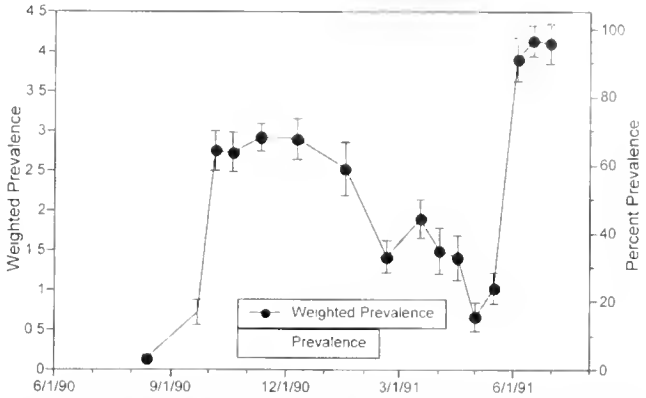

Figure 1. Seasonal pattern of *Perkinsus marinus* prevalence (percent) and weighted prevalence (mean ± se) during the study period. Each data point represents 40 oysters.

increase occurred from mid October to mid November 1990, coincident with the initial development of *P. marinus* infections, and a decrease in size was measured in late winter that may have been a combination of no growth and chipping of the shell edge. Rapid shell growth resumed in early April and continued through the early summer of 1991; even after infection intensities had become heavy (Fig. 1, Fig. 3). There was a marginally nonsignificant association between shell size and WP during the autumn, 1990, infection peak ($p = 0.077$, $r^2 = 0.011$); the correlation for the June 1991 peak was significant, although weak ($p = 0.015$, $r^2 = 0.054$). At both times, however, the correlation coefficient was positive, indicating that the larger oysters tended to be the more heavily infected.

The GI decreased from a peak of 0.55 to about 0.15 as spawning progressed during the summer of 1990, before the *P. marinus* epizootic began. It began to rise again in mid April 1991 and peaked in mid to late May at 0.40, then declined through June, when our sampling ended (Fig. 4). Overall, we found a strong seasonal variation in the effects of *P. marinus* infection on the reproduction and condition of oysters (Table 2). During the gamete resorption phase of late summer 1990, most oysters were not de-

TABLE 1.

Prevalence and intensity of *Perkinsus marinus* infection in oysters sampled from lower Delaware Bay from August 1990 to late June 1991. Prevalence is the percentage of oysters infected in a given period. Intensity is the percentage and number of oysters in a period that had a given intensity of infection (Ray/Mackin ratings).

Stage of the Reproductive Cycle (Sample Dates)	Total N	% Infected	No Infection (0) % (N)	Light Infection (0.5 & 1) % (N)	Moderate Infection (2 & 3) % (N)	Heavy Infection (4) % (N)	Very Heavy Infection (5) % (N)
Gamete Reabsorption (8/13 to 9/22/90)	62	50	50 (31)	40 (25)	8 (5)	0	2 (1)
No Reproduction (10/6 to 12/10/90)	241	84	16 (39)	13 (32)	28 (67)	15 (36)	28 (67)
Early Gametogenesis (1/17 to 4/2/91)	157	87	13 (21)	45 (71)	17 (27)	11 (18)	13 (20)
Late Gametogenesis (4/17 to 5/1/91)	80	61	39 (31)	40 (32)	10 (8)	4 (3)	8 (6)
Early Spawning (5/16 to 6/4/91)	79	84	16 (13)	28 (22)	16 (13)	8 (6)	32 (25)
Late Spawning (6/16 to 6/30/91)	60	100	0	3 (2)	23 (14)	18 (11)	53 (33)

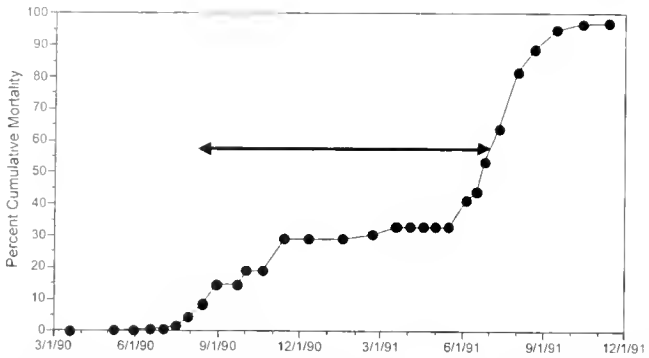

Figure 2. Cumulative percent mortality of oysters before, during, and after the study period. The sampling period is indicated by the double ended arrow.

tectably infected or had only light infections (Table 1). Nevertheless, there was a significant overall effect of infection on the GI, caused by the few oysters with moderate to heavy infections. The average GI of these individuals was half that of the uninfected oysters (Fig. 5). We found no significant effects on the other reproductive measures or on the condition index (Table 2, Fig. 6, Fig. 7, Fig. 8).

During the fall–early-winter period there was no obvious reproductive activity as judged by the absence of gametes and the very low quantity of gametogenic tissue. Nevertheless, we measured a significant effect of parasite burden on both GI and CI (Table 2). Again, it was the worst infected oysters that were affected. The average GI of heavily infected oysters (Ray/Mackin rating = 5) was two–thirds that of uninfected oysters and the CI was three-quarters (Fig. 6). There was no significant effect on the proportion of gametogenic tissue (Table 2, Fig. 7).

In both the gamete-resorption and the reproductive–inactivity periods, 2-way ANOVAs showed a significant effect of sample date, indicating that the measures of reproduction and condition changed over time within these periods. There were no significant interactions of parasite burdens and sample dates in any of the analyses, however, indicating that the relative pattern of the effects of these two factors did not change.

During the winter period of early gametogenesis there was a significant effect of parasite burden on all measures of reproduction and the CI (Table 2, Fig. 5, Fig. 6, Fig. 8). The average GI in heavily infected oysters was half that of uninfected oysters, but there was a trend toward smaller indices in oysters beginning with category 3–4 infections (Fig. 5). Similarly, the average proportion of gametogenic tissue and gametes was also about half that of uninfected oysters (Fig. 7, Fig. 8), with a suggestion that gamete production was impaired in oysters with relatively light infections (Fig. 7). Even though average parasite burdens declined, as is typical during the spring (Fig. 1), reproductive activity during the period of late gametogenesis was severely impaired by *P. marinus*. All measures of reproduction, as well as the CI, were affected (Table 2). The clearest impact was on the GI, which was significantly depressed at all infection levels (Fig. 5). The gonads of oysters with category 2–5 infections occupied, on average, only one-third the proportional area as those of uninfected or lightly infected individuals. The average proportion of gametogenic tissue and gametes in oysters with categories 4 and 5 infections, was only a quarter of that in uninfected oysters (Figs. 7 and 8). The CI of the most heavily infected oysters was two-thirds that of uninfected oysters (Fig. 6).

Finally, during both the early spawning period of late May to early June and the later spawning period of mid-June to late June there were no detectable effects of parasite burdens on any of the reproductive indices or on CI (Table 2, Fig. 5, Fig. 6, Fig. 7, Fig. 8). There were marginally nonsignificant trends toward less gametogenic tissue (Table 2, $p = 0.058$) and fewer gametes (Table 2, $p = 0.081$) during the later spawning period, but statistical power was lost due to small sample sizes in the uninfected and lightly infected categories (Fig. 7, Fig. 8).

DISCUSSION

One of the most common sublethal effects of parasites on their host is a reduction in growth and reproduction (Thompson 1983, Price et al. 1986, Ford & Figueras 1988, Forbes 1993, Ford & Tripp 1996). Although an inhibitory effect of *P. marinus* on oyster growth has been reported (Menzel & Hopkins 1955, Paynter & Burreson 1991), the consistent quantification of an impact on reproduction has been elusive to date (Choi et al. 1994, Kennedy et al. 1995, Paynter 1996). We found clear evidence that *P. marinus* infection had a significantly negative effect on relative gonadal size, and on the proportions of gametogenic tissue and gametes in the gonad. Detection of an effect depended on when during the disease and reproductive cycles the sample was taken, the type of

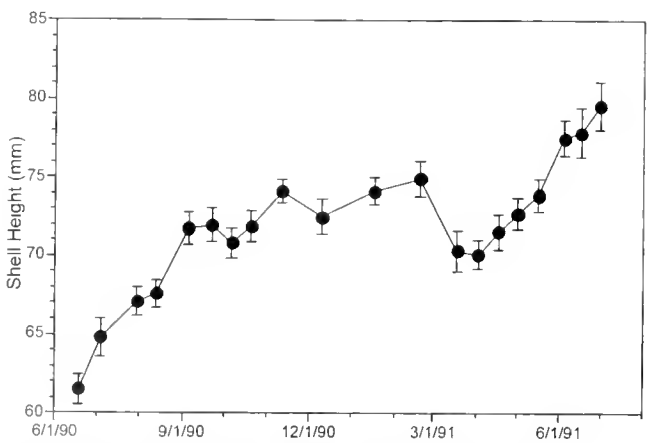

Figure 3. Shell growth of oysters during study period. Each point represents the mean (± se) shell size of 400 individuals.

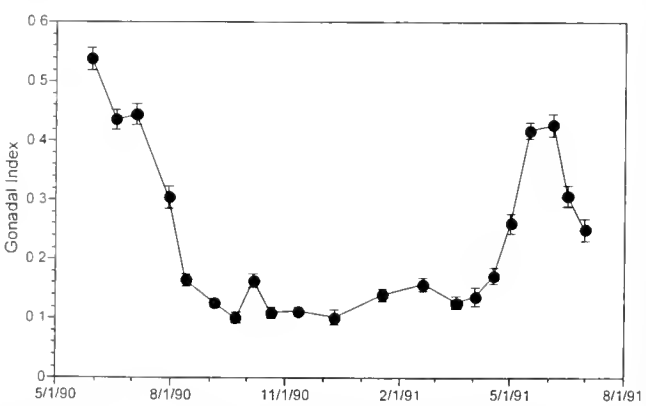

Figure 4. Seasonal changes in the gonadal index before and during the study period. Each point represents the mean (± se) of 40 individuals.

TABLE 2.

Results of 2-way ANOVA. Sample date was a fixed factor and intensity of *P. marinus* on the Ray/Mackin scale was a random factor. There were no significant interactions of parasite burden and sample date for any of the analyses. Parasite effect *P* values significant at <0.05 level are indicated in bold.

Stage of the Reproductive Cycle	ANOVA Model Factors (N)	*P* Values from 2-way ANOVAs			
		% Gonad Area	Condition Index	% Gametogenic Tissue	% Gametes
Gamete Reabsorption (8/13 to 9/22/90)	Parasites (4)	**0.018**	0.394	0.848	None
	Dates (2)	0.026	0.002	0.019	
	Total N (62)				
No Reproductive Activity (10/6 to 12/10/90)	Parasites (6)	**0.006**	**<0.001**	0.110	None
	Dates (4)	<0.001	0.004	0.041	
	Total N (241)				
Early Gametogenesis (1/17 to 4/2/91)	Parasites (7)	**0.004**	**0.002**	**0.008**	0.036
	Dates (4)	0.124	<0.001	0.468	0.188
	Total N (157)				
Late Gametogenesis (4/17 to 5/1/91)	Parasites (5)	**<0.001**	**<0.001**	**0.013**	**0.048**
	Dates (2)	0.224	0.795	0.578	0.708
	Total N (80)				
Early Spawning (5/16 to 6/4/91)	Parasites (5)	0.260	0.114	0.129	0.178
	Dates (2)	0.917	0.933	0.232	0.257
	Total N (79)				
Late Spawning (6/16 to 6/30/91)	Parasites (4)	0.917	0.898	*0.058*	*0.081*
	Dates (2)	0.295	0.262	0.877	0.806
	Total N (60)				

measurement made, and the parasite burdens of the oysters examined. The effect was most pronounced during the gametogenic phases of the reproductive cycle, but an effect on relative gonadal size could also be detected during the gamete resorption and overwinter inactivity periods. Interestingly, by the time oysters were reproductively mature and ready to spawn, we were no longer able to measure an effect of the parasite, although later in the spawning period there was again a trend (marginally nonsignificant) of decreasing proportion of gametes with increasing *P. marinus* burdens.

The effect of *P. marinus* increased with increasing infection intensity and was generally statistically significant only in oysters with heavy infections (Ray/Mackin rating of 4 or 5). During gametogenesis, heavy infections reduced the relative gonadal size to an average one-third to one-half that of uninfected or lightly infected oysters, and in some individuals with advanced infections, the gonad could not be detected at all. The other major disease agent of eastern oysters, *H. nelsoni*, impairs reproduction to a similar extent. Barber et al. (1988) reported that the Gonadal Index (GI) of oysters heavily infected with *H. nelsoni* averaged 50% or less that of uninfected animals and in several samples the gonadal area could not be measured.

The reduction in GI, by itself, reflected a potentially serious impact of *P. marinus* infection; however, the additional measures that we obtained in this study, CI and the proportion of the gonad occupied by gametes, indicate that the negative effect of *P. marinus* infection was considerably more severe than the GI alone would indicate. For example, the most extreme inhibition was measured during the period of rapid gametogenesis in the spring, when oysters having infections rated 4 or 5 had one-third the GI, with only one quarter of the gonadal area being filled with gametes or gametogenic tissue. Thus, the two-dimensional cross-sectional body area measured, contained fewer than 10% as many gametes as did the cross-sectional area measured in uninfected individuals. Further, the CI (directly correlated with cross-sectional body area, $p < 0.0001$) indicated that heavily infected oysters had only two thirds as much soft tissue as uninfected animals. Thus, the cross-sectional area, on which the proportional measures are based, was itself considerably diminished with infection, further reducing the absolute number of gametes produced.

The negative effect of *P. marinus* infection on reproduction and condition was clear during the period of gametogenesis, but it was absent or inconsistent for the same measurements made during other phases of the reproductive cycle. To understand the variable effects of the parasite at different seasons, it is necessary to consider the metabolic changes experienced by both the host and the parasite, and how they may interact, over the course of a year.

As oysters were ending the reproductive season in the late summer of 1990, all measures of reproduction were decreasing and *P. marinus* infections were beginning to intensify. The significant ANOVA effect of parasitism at this period was produced by the few infections that reached the moderate to heavy stage (Table 1) and depressed the GI to about half that of oysters with lighter or no detectable infections. The same trend continued into the fall and early winter, as many more oysters became heavily infected and by which time the impact of parasitism was measurable in reduced CI. Even though gamete production was not occurring, the relative area occupied by gonadal follicles was diminished. Glycogen, which is being stored in the late summer and fall, and which is measured by CI, is stored in the cells surrounding each follicle. The relative "fullness" of the storage cells is likely to have affected the measurement of gonadal size. That is, the GI is likely to have been relatively large in animals with high glycogen (CI), because it would include expanded storage cells. Because the amount of gametogenic material produced is directly related to the quantity of glycogen stored before gametogenesis (Loosanoff 1965), the impact of *P. marinus* on reproduction may begin as early as the late summer and fall of the preceding year. Kennedy et al. (1995) also

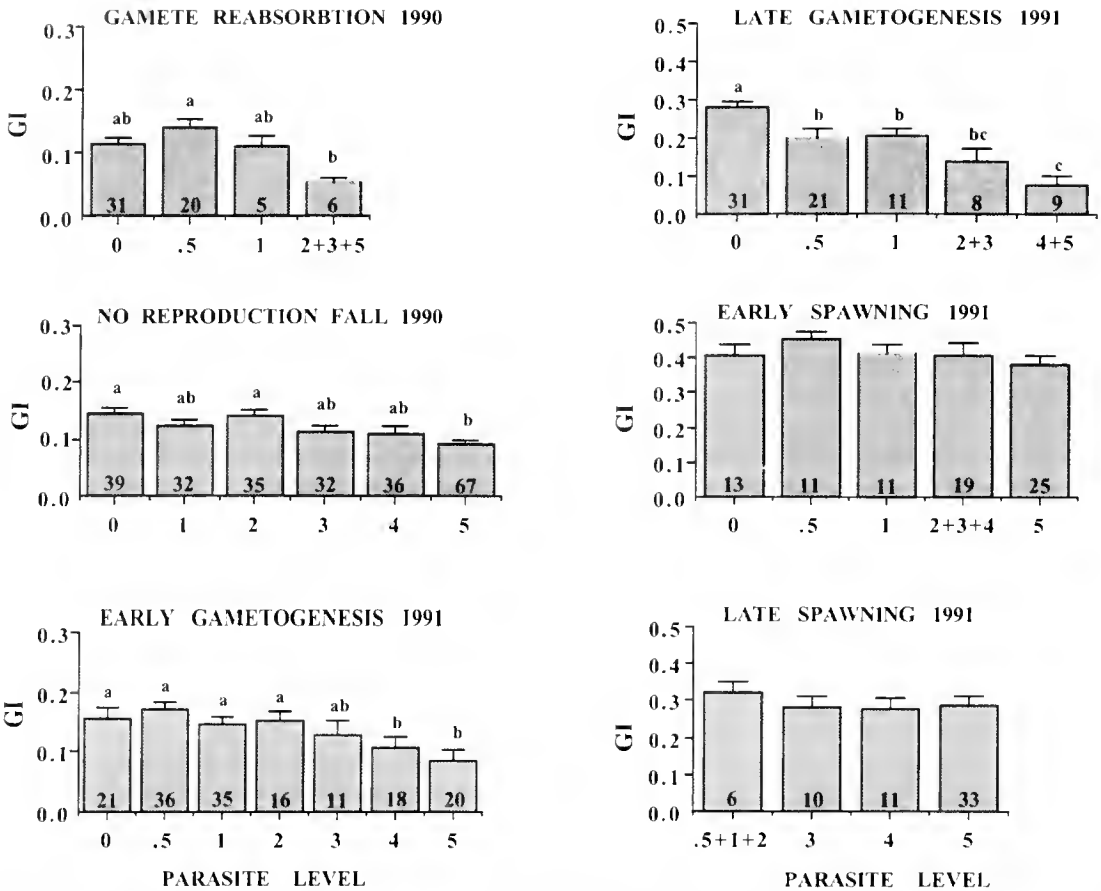

Figure 5. Mean (± se) gonadal index for oysters, plotted according to *Perkinsus marinus* infection category, during all 6 reproductive cycle periods. When there were small sample sizes, parasite levels were pooled as indicated. Letters above the columns indicate statistically significant differences ($p < 0.05$).

hypothesized that reproductive capacity would be impaired if oysters had been parasitized the previous year, but were unable to confirm it.

Probably the most surprising finding was the lack of measurable effect of *P. marinus* on the reproductive and condition indices at the time of maximum reproductive condition (mid-May through early June) when the mean GI was 0.4 or more, and 80% of the gonad was occupied by gametes in oysters of all infection levels, including those patently uninfected. Given the very serious effect that parasitism had had during the gametogenic period, just a few weeks earlier, this almost sudden lack of effect is puzzling. Death of heavily infected oysters was not responsible because spring mortality did not begin until after this peak condition had already been reached (Figs. 2 and 3). Further, both the early and late spawning periods, (from mid-May through the end of June) contained substantial numbers of oysters with advanced infections that showed no statistical effect of infection on gonadal indices. The data therefore indicate that the oysters showing a pronounced effect of parasitism during the gametogenesis were the same individuals that showed essentially no effect of parasitism several weeks later, when they were reproductively mature.

To help explain this phenomenon, it important to remember that the period in question is one of very rapid change. The spring phytoplankton bloom provides ample food (Ford 1986, Powell et al. 1997), oyster growth rates are rapid, as is gametogenesis. At the same time, infection intensities are diminishing within individual oysters. In our study, weighted prevalence reached a low point at the beginning of May 1991, coincident with the beginning of the most rapid increase in gonadal size. Only about 10% of the oysters had advanced (Ray/Mackin stage 4 and 5) infections at this time. Parasite burdens remained low through mid-May when the gonadal indices peaked. Consequently, during the period of most rapid gametogenesis and maturation, as measured by the doubling of the GI between mid-April and early June, *P. marinus* infection loads were not only very low, but had been decreasing for several months. Accordingly, it can be argued that this respite from the metabolic demands and physiological disruptions of the parasite, during a period of generally good food conditions, allowed the oysters to recover, build gonad, and spawn before parasite loads again reached the point at which they imposed a metabolic burden. Interestingly, Ford and Figueras (1988) reported a similar event in oysters infected with *H. nelsoni*. In that case, infection intensity diminished quickly in early June, much later than the gradual decrease recorded for *P. marinus*, but apparently in enough time for oysters to recover, produce gametes, and spawn later in the summer.

The apparent rapid recovery of sick oysters is remarkable, but reproduction did not entirely escape the effects of *P. marinus* infection during the spawning period. There was a trend towards a lower proportion of gametogenic tissues (nearly all gametes at this time) during the late spawning period, after infection levels had peaked. Also, *P. marinus* could also have been responsible for the

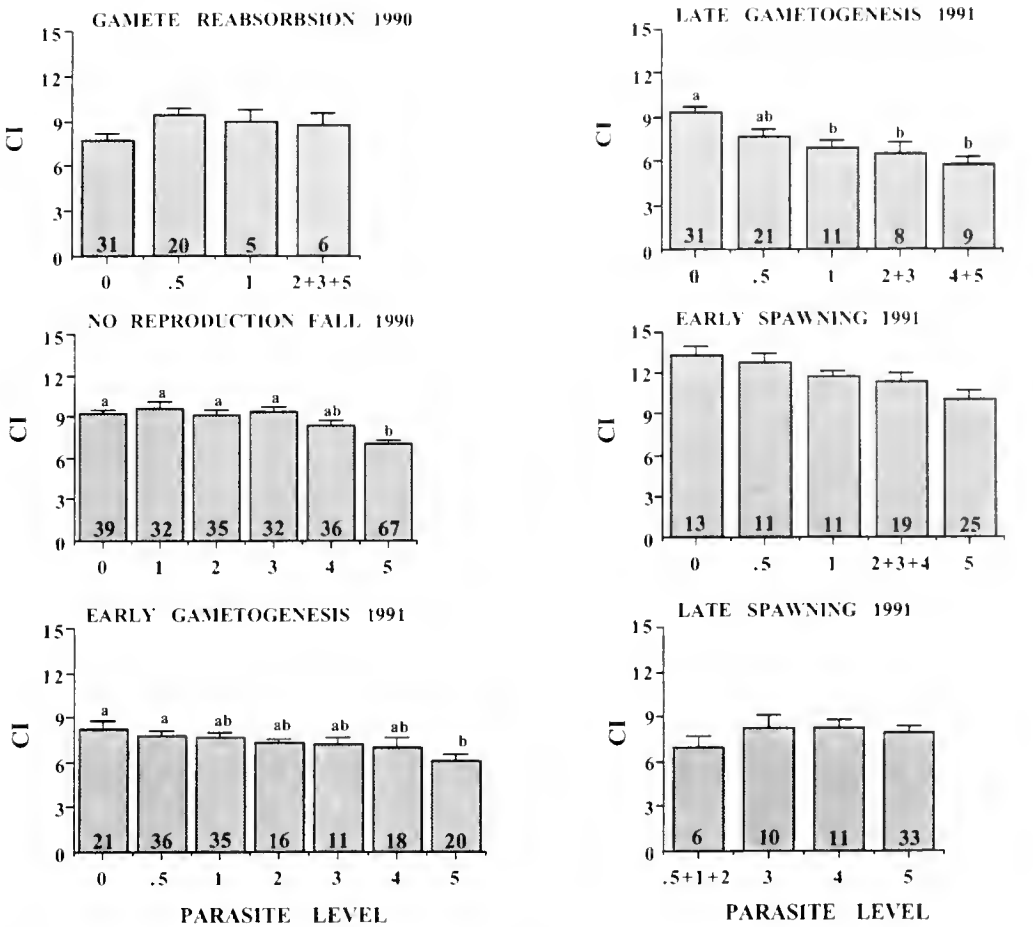

Figure 6. Mean (± se) present condition index for oysters, plotted according to *Perkinsus marinus* infection category, during all 6 reproductive cycle periods. When parasite levels were pooled due to small sample sizes it is indicated. Letters above the columns indicate statistically significant differences ($p < 0.05$).

overall lower GI in 1991 compared to 1990, before the epizootic, although year-to-year variability in food and other factors cannot be excluded. Since we did not measure egg size, there is a possibility that the infected oysters had smaller eggs, which would have diminished their chances of survival during the larval stage (see Gabbott 1983). Previous studies, however, found no reduction in egg size associated with either *P. marinus* or *H. nelsoni* infection (Barber et al. 1988, Kennedy et al. 1995).

We also found significant negative effects of *P. marinus* on oyster Condition Index (CI), consistent with previous research (Ray et al. 1953, Mackin 1962, Paynter & Burreson 1991), but not at all seasons. The effect was measurable beginning in autumn, as oysters stored glycogen, and was observed throughout the spring period during which time gametogenesis was progressing. It was absent, however, throughout the spawning period, probably for the same reason that there was no effect on the GI at this time.

These results fit in well with recent ideas and models of oyster populations that describe how the interaction *P. marinus* and the oysters may vary seasonally (Hofmann et al. 1992, Powell et al. 1994, and Hofmann et al. 1995). Oyster growth and allocation to reproduction are a balance between food supply and competing energy requirements (Hofmann et al. 1992, Thompson et al. 1996). Our results suggest that the metabolic demands of *P. marinus* (Choi et al. 1994) may begin in the fall, as nutrient reserves are being stored, and persist into the spring. As noted by others, the gametogenic period is stressful for many bivalves as in many cases, especially in more northern populations, as they are relying on stored food (Loosanoff 1942, Bayne & Newell 1983, Thompson et al. 1996). Our data are consistent with this view. The clear association between *P. marinus* and impaired gametogenesis during early spring suggests that oysters were relying on reserves stored in the autumn, which had been diminished in proportion to infection level, to begin gametogenesis. The ability of infected oysters to develop mature gonads later in the spring may reflect the direct conversion of food, rather than stored energy, into gametes. It is relevant that Newell et al. (1994) found little or no effect of *P. marinus* on feeding rates or assimilation efficiency indicating that infected oysters would be as able as uninfected individuals to obtain and assimilate the nutrients present in spring blooms.

Our data showing little or no effect of *P. marinus* at the time of maximum reproductive condition and during spawning is at variance with those of Kennedy et al. (1995). These authors followed two oyster populations in upper Chesapeake Bay and found the only measurable impact of *P. marinus* to be at spawning, when we found none. The Chesapeake Bay relationship was true at one site only, however. No effect of parasitism was found, at any time, at the second location. Consequently, site-specific factors are likely to have a large influence on the relationship between parasitism, reproduction, and somatic growth and these factors are probably the ones influencing the acquisition of energy. For instance, math-

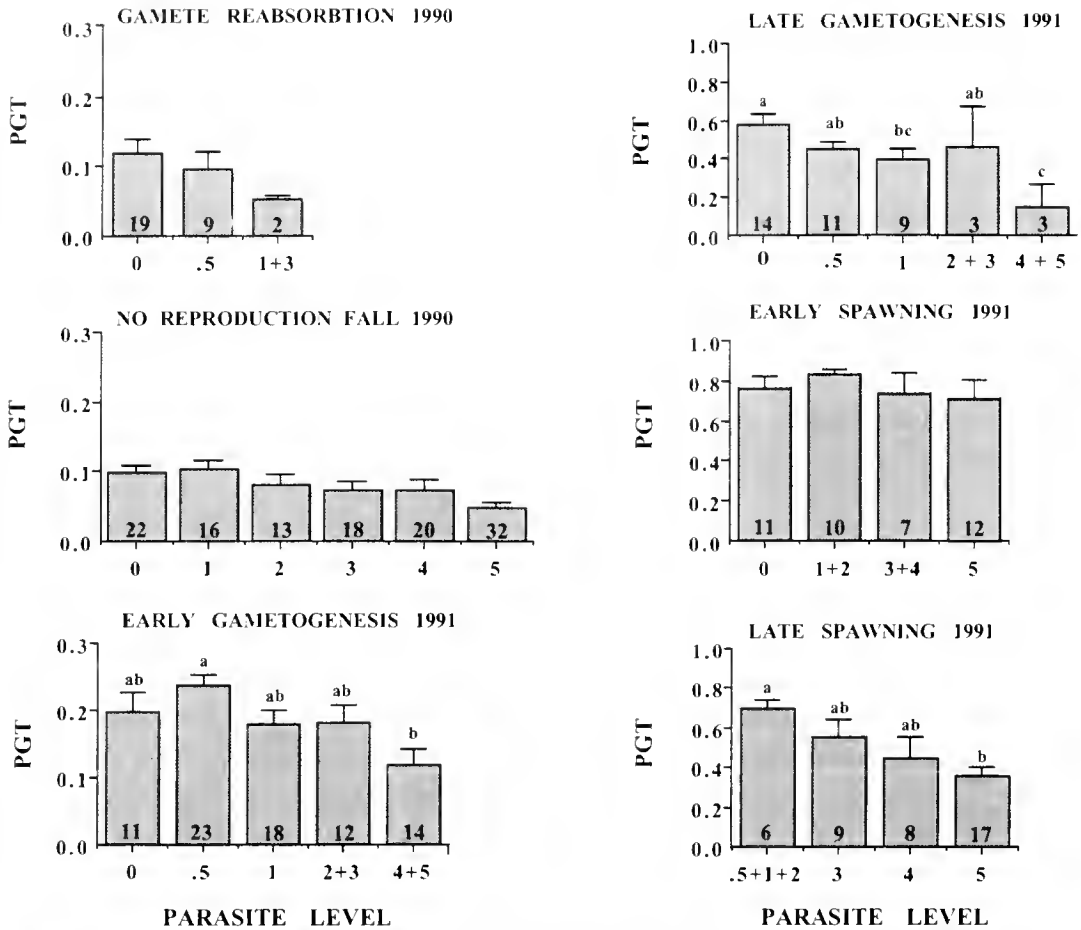

Figure 7. Mean (± se) percent gametogenic tissue for oysters, plotted according to *Perkinsus marinus* infection category, during all 6 reproductive cycle periods. When parasite levels were pooled due to small sample sizes it is indicated. Letters above the columns indicate statistically significant differences ($p < 0.05$).

ematical modeling suggests that the timing and extent of phytoplankton blooms can play an important role in the outcome of oyster–parasite relationships (Powell et al. 1996, Ford et al. 1996) and these are likely to vary widely, even in the same estuary system (Powell et al. 1997). Temperature differences, which would affect the timing of infection and reproductive cycles, would also be critical.

Menzel and Hopkins (1955) were the first to recorded an impact of *P. marinus* infection on oyster growth. By following individually marked oysters, they documented a negative correlation between parasite burdens and shell growth. More recently, Paynter and Burreson (1991) reported that even very light infections in a population of oysters were sufficient to markedly slow the average growth rate. We, too, recorded a decrease in shell growth rate coincident with the onset and development of infections in the autumn. When growth resumed in the spring, infection intensities were apparently too low to affect shell deposition, but rapid growth continued even after parasite loads became high. This unexpected result did not occur because heavily infected oysters died, as they tended to be larger, not smaller, than lightly infected animals. Rather, it may be due to the abundant food supply at the Cape Shore test site, which has always been an excellent location for oyster growth (Dittman et al. 1998). High phytoplankton levels may have provided sufficient nutrients to fuel gamete production and somatic growth, as well as parasite growth. Whether "recovery" would have occurred under less favorable conditions than we hypothesize is unknown. An interesting question is why has it been so hard to consistently quantify the sublethal effects of *P. marinus* infections on reproduction. Some studies suffered from low sample

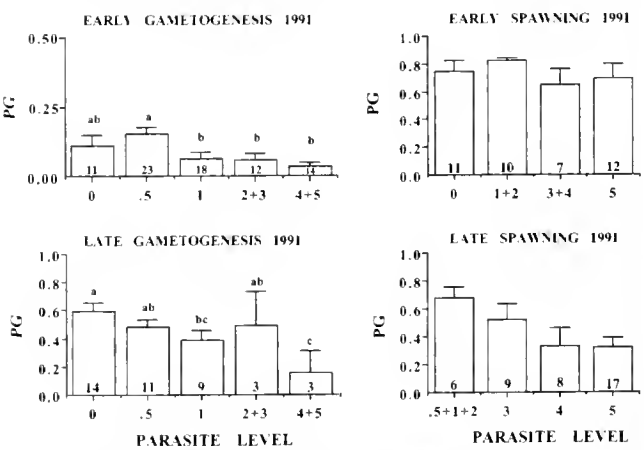

Figure 8. Mean (± se) percent gametes for oysters, plotted according to *Perkinsus marinus* infection category, during all 6 reproductive cycle periods. When parasite levels were pooled due to small sample sizes it is indicated. Letters above the columns indicate statistically significant differences.

sizes for the amount of reproductive and parasite level variation observed and there were scant data on the history of the oyster populations and their previous exposure to disease. Also, it is clear that for the most part, *P. marinus* intensities do not impair nutrient storage or reproduction until they become heavy (Ray/Mackin stages 4–5). Unless samples contain oysters with advanced infections, an effect of *P. marinus* on reproduction will be difficult to detect.

Our study has shown the need to consider the seasonal cycles of both protagonists in attempting to assess the metabolic effects of an endoparasite on its invertebrate host. Samples taken at a single season may fail to show an effect that is clear at another, nor will they provide the history of the populations needed to interpret any given sample result. For instance, the very drastic negative effect on reproduction predicted during the gametogenic phase did not materialize at the time of spawning because a period of diminishing parasite burdens coincided with gamete maturation. Our investigation was also helped because all oysters were of known age and genetic history, were reared in a common environment, and were exposed to the disease agent at the same time. All of these factors and an experiment that involved sampling throughout the host's reproductive cycle and the parasite's infection cycle allowed us to detect and interpret the effects of infection intensity on oyster reproduction more easily than would otherwise have been possible.

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THE EFFECTS OF A REGULATORY GEAR RESTRICTION ON THE RECRUITING YEAR CLASS IN THE SEA SCALLOP, *PLACOPECTEN MAGELLANICUS* (GMELIN, 1791), FISHERY

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ABSTRACT In 1994, Amendment 4 to the sea scallop (*Placopecten magellanicus*) fishery management plan was adopted, which restricted fishing effort by controlling vessel days at sea, crew size, and gear size. Dredge ring size was increased from 76.2 mm (3.0″) to 82.6 mm (3.25″) in March 1994, and again to 88.9 mm (3.5″) in January 1996 to increase the age of entry of scallops into the fishery. Between June 1994 and April 1995, four trips were taken on commercial scallop vessels in the western mid-Atlantic to determine harvest efficiency of 88.9-mm dredge rings relative to 82.6-mm dredge rings used in the fishery at the time. Our study focused on the abundant and nearly ubiquitous 3-year-old, 1990 year class. At the time, individuals in this year class were approaching the size (70 mm) of both full recruitment to the gear and recruitment into the fishery. Relative harvest efficiency of this year class ranged from 60% to 72% over the study period. The 88.9-mm rings were found to be 90% efficient when scallops had grown to a size of 100–105 mm. The 88.9-mm ring dredge would therefore delay full recruitment of the 1990 year class for almost 1 y relative to the 82.6-mm ring dredge. Analysis shows that this delay could increase yield-per-recruit by almost 10% and spawning stock biomass by 40% to 60%. Benefits of the gear modifications will only be fully realized when used in conjunction with other measures that reduce or stabilize fishing effort.

KEY WORDS: sea scallop, relative harvest efficiency, ring size, *Placopecten magellanicus*

INTRODUCTION

The fishery for the Atlantic sea scallop *Placopecten magellanicus* (Gmelin 1791) began in the United States in the mid-Atlantic region in the 1920s and expanded in the 1930s with the discovery of large concentrations of scallops on Georges Bank (Bourne 1964, Serchuk et al. 1979). Commercial landings increased rapidly following World War II, and reached a peak of 17,174 metric tons of meats valued at over $145 million in 1990 (NOAA 1991).

Despite high levels of exploitation and fluctuating annual landings, the first fishery management plan (FMP) for the sea scallop was not in place until 1982 (NEFMC 1982). Objectives of the original sea scallop FMP were to be achieved by controlling the age at entry of the scallops into the fishery. An average meat count restriction of not more than 30 meats per pound (MPP) was enforced for vessels that landed shucked meats. For vessels landing shell stock, a minimum shell size was set at 88.9 mm (3.5 inches).

Several amendments were made to the FMP to correct perceived inadequacies and enforcement difficulties; however, the FMP remained relatively unchanged until 1994. Many of these problems with the original sea scallop FMP are discussed by Naidu (1987), Shumway and Schick (1987), Smolowitz and Serchuk (1987, 1988), DuPaul et al. (1989), Kirkley and DuPaul (1989), and Schmitzer et al. (1991). One major problem of the 1982 FMP was the attempt to manage a fishery that extends over a wide geographic area based on a single parameter of the animal, the adductor muscle size. Size of the adductor muscle has been found to vary widely between resource areas, water depths, seasons, and within the reproductive cycle (Haynes 1966, Shumway & Schick 1987, DuPaul et al. 1989), and this confounded management efforts.

In March 1994, Amendment 4 to the sea scallop FMP was implemented to replace existing MPP restrictions. Amendment 4 restricted fishing effort by limiting vessel days at sea, crew size, the size of the fishing gear, and entry of new vessels into the fishery. Age at entry controls were implicitly imposed by increasing the size of the scallop dredge rings and the mesh of the scallop trawl nets (NEFMC 1993). The primary gear restriction of Amendment 4 increased the minimum ring size of the dredge from 76.2 mm (3.0″) to 82.6 mm (3.25″) for 1994 and 1995, and to 88.9 mm (3.5″) beginning January 1, 1996. Previous gear trials have shown that increasing dredge ring size or trawl mesh size allows an increased escapement of smaller-sized scallops (Medcof 1952, Bourne 1965, Smolowitz & Serchuk 1988, DuPaul et al. 1988, DuPaul et al. 1989).

In this paper we present an assessment of the effects of an increase in dredge ring size on aspects of selectivity, harvesting efficiency, and delay of entry into the fishery. The study targeted the very large 1990 year class of scallops (NEFSC 1993). We found that an increase in scallop dredge ring size will increase escapement of small, pre-recruit scallops less than 70 mm and will delay entry of scallops greater than 70 mm into the fishery. This regulatory measure could lead to substantial increases in yield and spawning potential of specific year classes of scallops.

MATERIALS AND METHODS

Data Collection

Data for this study were collected during four trips aboard commercial scallop vessels (June, August, and November 1994 and April 1995), each lasting 7 to 14 days. Sampling was conducted aboard the fishing vessel (F/V) *Carolina Breeze* and the F/V *Stephanie B* in the Delaware/Maryland/Virginia region of the western mid-Atlantic (NAFO statistical area 6, Fig. 1). Both vessels are approximately 23.01 m (75.5 feet) in length. Catches were sampled from 209 of the 759 tows conducted with the experimental gear.

The fishing gear used during these experiments was the standard offshore New Bedford style scallop dredge used by most vessels in the fishery. Posgay (1957) provides a general overview of the gear, and a more detailed description is given in Bourne (1964). For each trip, the control gear was constructed from 82.6-mm (3.25″) rings, the size used in the fishery at the time of the study. The experimental gear was constructed from 88.9-mm (3.5″)

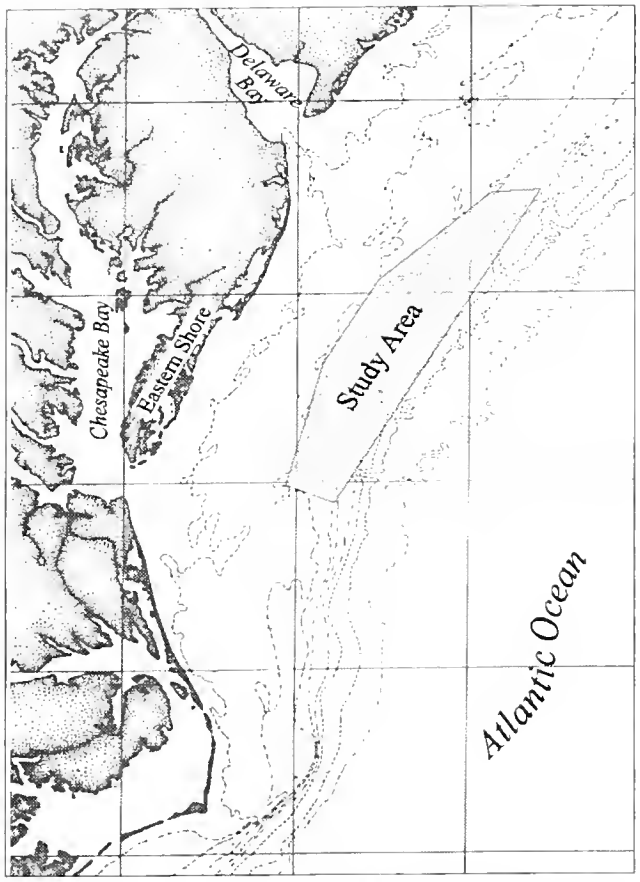

Figure 1. Location of sampling efforts in the Mid-atlantic region.

rings. Both vessels used are capable of towing two dredges simultaneously. Since studies have shown that there is no statistical difference in catch for dredges towed in pairs (Bourne 1965, DuPaul et al. 1989), the control and experimental gears were towed simultaneously. The *Carolina Breeze* towed two 4.27-m-(14-foot) wide dredges, and the *Stephanie B* towed two 3.96-m-(13-foot) wide dredges.

Data collection methodology was similar to that of Bourne (1965) and DuPaul et al. (1989). Sampling procedures were designed to allow usual commercial operating procedures, except that catches from the two dredges were kept separate throughout the trip. For sampled tows, the crew was allowed to cull out the commercial-sized scallops. The remaining bycatch was then sorted to retrieve undersized or discarded scallops. Up to two baskets (1 basket is approximately 1.5 bushels) of commercial-sized scallops, and at least a portion of the discards were retained from each dredge for shell height frequency sampling. Shell height, the maximum distance from the umbo to the ventral margin of the shell, was measured to the nearest 5-mm interval using National Marine Fisheries Service scallop measuring boards.

Six one-basket samples of scallop shell stock were obtained from commercial scallop vessels between February and May 1994 to collect shell height and meat weight data. Each sample represented a single trip by a single vessel. When these scallops were shucked, the upper (left) valve was measured to the nearest millimeter using a measuring board, and the respective meat was weighed to the nearest 0.1 g using an Ohaus CT 600 electronic scale.

Data Analysis

To compare catches from the different trips, the data were standardized to a common unit of effort equal to 50 h of tow time using a 3.96-m wide dredge using the following equations:

$$N_{ij} = (n_{ij}/b_j) \times B_j \tag{1}$$

$$SPH_{ij} = N_{ij} \Big/ \left(T_j/_{60}\right) \tag{2}$$

$$SC_i = \sum_{j=1}^{ST} SPH_i \Big/ \left(ST/_{50}\right) \tag{3}$$

where N_{ij} = the total number of scallops at shell height i in tow j; n_{ij} = the number of scallops sampled at shell height i in tow j; b_j = the number of baskets sampled in tow j; B_j = the total number of baskets in tow j; SPH_{ij} = the number of scallops caught per hour at shell height i in tow j; T_j = tow time in minutes of tow j; SC_i = standardized number of scallops caught at shell height i for the trip; and ST = the number of tows sampled during the trip.

For data from the trip using the 4.27-m-wide dredges, n_{ij} was multiplied by 13/14 to standardize to a 3.96-m-wide dredge before using the above equations.

Harvest efficiency of scallops from the 1990 year class was determined for the 88.9-mm ring dredge relative to the 82.6-mm ring dredge for each 5-mm shell height interval within the year class and for the year class as a whole. This was done by dividing the number of scallops caught in the larger ring dredge by the number caught in the smaller ring dredge. Efficiency estimates for the individual shell height intervals were then smoothed using a moving geometric mean of three (Pope et al. 1975, Serchuk & Smolowitz 1980, DuPaul et al. 1989).

The range of the shell height intervals used for the year class was found using the Petersen method (Jearld 1983). Year classes were distinguished by different peaks in the shell height frequency distributions for scallops from all trips, except the April 1995 trip. In April 1995, the 1991 year class distribution overlapped slightly with the 1990 year class distribution. The 1990 year class was delineated by finding the modal shell height of the year class and visually estimating the right tail of the distribution. It was then assumed that the number of size classes present on each side of the mode was the same.

The conventional allometric or shell height:meat weight model was estimated using SAS software (version 6.09). A log-log transformation was necessary to allow estimation of the relation using ordinary least squares. A random effects model was run, with time of collection being the random variable, in order to combine the samples and obtain a common shell height:meat weight relationship. The model can be expressed as:

$$\ln(MW) = \ln(a) + b \times \ln(SH) + u \tag{4}$$

where "ln" is the natural logarithm, MW and SH are meat weight and shell height respectively, and the error term u is $N(0, \sigma^2)$.

Scallop growth was estimated by applying an exponential growth model to catch data collected during different trips, including data from a trip taken in November 1993 (DuPaul & Kirkley 1994a, DuPaul & Kirkley 1994b). It was during the November 1993 trip that the 1990 year class first started to recruit to the 76.2-mm ring dredge used at the time (DuPaul & Kirkley 1994a). The growth model can be expressed as

$$SH_t = SH_0 \times \exp[G \times (t - t_0)] \quad (5)$$

where SH_t is shell height at time t, SH_0 is shell height at time 0, and G is the exponential growth coefficient.

The mean shell height of the specified age class from each of the trips was plotted against number of days relative to the first sampling trip. It was assumed that daily growth of scallops during each trip was minimal, and that for each trip, all scallops were collected on one day. The first day of each sampling trip was arbitrarily designated the collection day. All increments in days were counted from the first day of the first trip to the first day of each successive trip.

Results of the efficiency, growth, and shell height:meat weight calculations were used to examine the effects increasing ring size to 88.9 mm might have on yield-per-recruit (YPR) of the fishery. The YPR calculations assume only scallops 70 mm and larger are retained by the fishermen. The June 1994 trip was used as a reference point, and the catch in the 82.6-mm ring dredge during that trip was assumed to be a representative sample of the shell height frequency distribution of the year class. Catch in the 82.6-mm ring dredge in June 1994 was multiplied by 10 to give an arbitrary initial abundance of the year class (N_0). Values of fishing mortality (F) and natural mortality (M) were set at $F = 1.5$ (NEFMC 1993) and $M = 0.1$ (Dickie 1955, Merrill & Posgay 1964) for a full year (0.75 and 0.05, respectively, over 6 mo). The year was broken into two 6-mo periods, and the equation

$$N_t = N_0 \times e^{-F \times t} \quad (6)$$

was applied to find population size at time $t = 6$ mo. At the end of each 6-mo period, the remaining population (N_t) was reduced by the natural mortality rate to find the N_0 for the next period. The growth coefficient was applied to the scallops comprising the new N_0 to obtain the new shell height frequency distribution. These calculations were repeated four times to simulate harvest over a 2-y period (0–6, 6–12, 12–18, and 18–24 mo). Catch for each period was estimated from $C = N_0 - N_t$. The shell height:meat weight model was applied to the harvest at each shell height for each repetition and was summed over all repetitions to finds landings for the entire period.

To estimate landings for the 88.9-mm ring dredge, similar methods were used except that the catch was adjusted by the relative efficiency at each shell height. N_t was found as $N_0 - C$, and then reduced by the natural mortality rate to give N_0 for the next period.

Spawning potential (in terms of overall fecundity of each animal) was estimated using residual reproductive value (RRV) (MacDonald 1984). RRV takes into consideration the probability of a scallop surviving between successive spawning events. The probability of survival between ages X and X + 1 is multiplied by the fecundity at age X + 1. Overall fecundity was found as the sum of fecundity at age X plus the RRV of fecundity at age X + 1.

RESULTS

Relative Efficiency

Harvest by each of the ring sizes during each of the trips is shown for each shell height standardized to 50 h of tow time in Table 1. Estimates of relative harvest efficiency by the 88.9-mm ring dredge ranged from 60% to 72% over the study period (Table 2). Examination of the efficiency estimates by shell height (Table 3) indicated that scallops from the 1990 year class would be captured with 90% to 100% relative efficiency when they reach 100 to 105 mm shell height. Harvest by the larger ring dredge exceeded harvest by the smaller ring dredge for several of the larger shell heights, but then decreased. With the exception of the November 1994 trip, relative efficiency increased for larger scallops. The unusually large decrease in efficiency in November 1994 is not readily explainable, but has been observed and documented during other gear trial studies (DuPaul et al. 1989).

Shell Height: Meat Weight and Growth

Six one-basket samples of scallop shell stock were obtained between February and May 1994 for use in shell height:meat weight analysis. Each sample represented a single trip by a single vessel. A random effects model was run, with time of collection being the random variable, in order to combine the samples and obtain a common shell height:meat weight relationship. The relationship found is shown below. The values under the equation are the t values for the coefficients.

$$\ln(MW) = -9.7776 + 2.6996 \times \ln(SH) \quad (-73.604)\ (98.136) \quad (7)$$

The estimates of the daily growth coefficient between successive trips are shown in Table 4. The overall estimate for the daily

TABLE 1.

Shell height frequency distributions for the 82.6- and 88.9-mm ring dredges for each trip, standardized to 50 h of tow time. The values in bold denote the 1990 year class. (Scallops smaller than 55 mm and larger than 110 mm caught by the gear are not shown.)

Shell Height (mm)	June 1994 (82.6 mm)	June 1994 (88.9 mm)	August 1994 (82.6 mm)	August 1994 (88.9 mm)	Nov 1994 (82.6 mm)	Nov 1994 (88.9 mm)	April 1995 (82.6 mm)	April 1995 (88.9 mm)
55–60	**22**	8	1069	545	870	502	989	610
60–65	**258**	**130**	**456**	**254**	**587**	**365**	1,876	1,161
65–70	**2,107**	**866**	**1,398**	**813**	**1,238**	**755**	**3,987**	**2,585**
70–75	**10,128**	**5,796**	**5,253**	**3,359**	**2,814**	**1,699**	**7,023**	**4,969**
75–80	**21,306**	**13,254**	**9,941**	**7,256**	**4,048**	**2,497**	**7,890**	**5,632**
80–85	**17,644**	**11,755**	**11,294**	**8,123**	**4,284**	**2,541**	**7,611**	**4,388**
85–90	**5,153**	**3,581**	**5,548**	**3,828**	**3,684**	**1,951**	**6,483**	**4,159**
90–95	**757**	**561**	**1,376**	**942**	**2,502**	**1,377**	**5,671**	**4,447**
95–100	**162**	**199**	**293**	**246**	**1,145**	**895**	**3,791**	**3,573**
100–105	162	171	**160**	**161**	**419**	**404**	**2,489**	**2,291**
105–110	149	163	201	175	169	167	**1,241**	**1,061**

TABLE 2.

Relative harvest efficiency estimates for the 1990 year class during each trip and for all trips combined.

Trip Date	Modal Shell Height (mm)	Catch in 82.6-mm Ring Dredge	Catch in 88.9-mm Ring Dredge	Relative Harvest Efficiency
June 1994	77.5	57,537	36,510	0.63
August 1994	82.5	35,719	24,982	0.70
November 1994	82.5	20,721	12,484	0.60
April 1995	92.5	42,653	30,916	0.72
Overall		47,596	31,542	0.66

growth coefficient ($G = 0.000736$) was used for all growth calculations in this paper.

YPR and Spawning Potential

Applying the shell height:meat weight relationship to the shell height frequency distribution of the 1990 year class observed in June 1994 results in approximately 490 kg of meats. Delaying harvest by 1 y (assuming knife-edge selectivity) would yield approximately 844 kg of meats, assuming $M = 0.1$ and relative efficiency is 90%. This is an increase in yield of more than 70%. The offshore scallop dredge, however, does not perform with knife-edge selectivity. When size-specific relative efficiency is considered over a 2-y period, yield from the larger ring dredge was approximately 8.3% more than the smaller ring dredge (Table 5). Based on analysis of cumulative fecundity of scallops ages 3 and 4 using residual reproductive values, increases in fecundity were estimated to range from 43% to 59% higher when the 88.9-mm ring dredge is used and more scallops are present to spawn at age 4 (Table 6).

DISCUSSION

When Amendment 4 became effective in March 1994, the sea scallop resource was considered to be overfished if spawning stock biomass (SSB) was below 5% of that of an unfished population. The optimum level of fishing mortality relative to the SSB threshold definition ($F_{5\%}$) was determined to be 0.97 (NEFMC 1993). A 1997 review of the sea scallop resource found that fishing mortality in the Mid-Atlantic had been above the overfishing definition since 1985 (NEFSC 1997). Higher than sustainable levels of fishing effort decreased the abundance of sea scallops, and subsequently, the number of exploitable year classes available to the fishery. This created the situation where the fishery is highly dependent on the recruiting year class (Serchuk et al. 1979, Brown 1987, NEFSC 1993, NEFMC 1993). When there are limited exploitable year classes, a fishery may be detrimentally affected by continued exploitation.

Gear regulations contained in Amendment 4 were implemented primarily to lower fishing mortality on small scallops and delay age at entry into the fishery. Data from this study suggest that the 88.9-mm rings will achieve these goals and also improve yield per recruit in the fishery and increase SSB of the scallop stock. The 88.9-mm dredge rings were anticipated to be an important factor in reducing fishing mortality to below the overfishing definition and meeting the management objectives, when used in conjunction with the other management measures.

Relative Selectivity, Efficiency, and Age at Entry

The year class targeted during this study was the largest year class on record to recruit to the Delaware/Maryland/Virginia region (NEFSC 1993). As many as 25 baskets (35–40 bushels) of these scallops were caught per tow in a single dredge during a sampling trip in November 1993 (DuPaul & Kirkley 1994a, Du-

TABLE 3.

Smoothed efficiency values by shell height for each trip and for all trips combined. The values in bold print denote the shell height range of the 1990 year class during each trip.

Shell Height (mm)	June 1994	August 1994	November 1994	April 1995	Overall
50–55	0.49	0.49	0.63	0.65	0.57
55–60	**0.49**	0.53	0.61	0.64	0.59
60–65	**0.42**	**0.55**	**0.60**	0.63	0.57
65–70	**0.49**	**0.59**	**0.61**	**0.66**	0.59
70–75	**0.53**	**0.65**	**0.61**	**0.69**	0.61
75–80	**0.62**	**0.69**	**0.60**	**0.66**	0.65
80–85	**0.66**	**0.71**	**0.58**	**0.64**	0.66
85–90	**0.70**	**0.70**	**0.56**	**0.66**	0.68
90–95	**0.86**	**0.73**	**0.61**	**0.78**	0.75
95–100	**0.99**	**0.83**	**0.72**	**0.88**	0.85
100–105	1.12	**0.90**	**0.91**	**0.91**	0.92
105–110	1.19	0.96	1.02	**0.88**	0.95
110–115	1.31	0.90	0.95	0.89	0.97
115–120	1.43	0.90	1.01	1.08	1.06
120–125	1.21	0.81	1.01	1.27	1.05

TABLE 4.

Estimates of the exponential growth parameter (G) for the 1990 year class for each trip and for all trips combined.

Trip Date	Mean Shell Height (mm)	Interval Between Trips (days)	Cumulative Interval (days)	Growth Coefficient ($\times 10^{-4}$)
November 1993	62.16		0	
June 1994	78.87	217	217	10.97
August 1994	80.17	67	284	2.44
November 1994	82.03	75	359	3.06
April 1995	92.50	181	540	6.64
Overall		540	540	7.36

Paul & Kirkley 1994b). The majority of these scallops were 60–65 mm and averaged 100 MPP. Larger individuals from this year class were already being retained by the fishermen (DuPaul & Kirkley 1994a).

Amendment 4 increased the ring size used in the scallop dredge initially from 76.2 to 82.6 mm, and subsequently to 88.9 mm. DuPaul and Kirkley (1994a, 1994b) have shown that the 82.6-mm ring dredge decreased efficiency of the scallop dredge (in terms of number of baskets caught) by 12% on soft bottom (sand and mud) to 45% on hard bottom of cobble, relative to the 76.2-mm ring dredge. During the present study, harvest efficiency of the 88.9-mm ring dredge relative to the 82.6-mm ring dredge (in terms of number of scallops caught) ranged from 60% to 72% (Table 2).

For this study, full recruitment (retention) to the experimental gear was considered to be reached when 90% efficiency relative to the 82.6-mm ring dredge was attained. For example, 90% to 100% relative efficiency was reached when scallops were 100–105 mm shell height (Table 3). Using the June 1994 modal shell height of 77.5 mm as a reference point, and by applying the estimated growth equation determined in this study, it would take the 1990 year class 346 days, or nearly a year to reach the size of 100 mm and thus be fully retained by the 88.9-mm ring dredge.

YPR

Many studies have examined the effects of delaying harvest as a means to increase YPR in terms of meat weight in the scallop fishery (e.g., Posgay 1958, Posgay 1962, Caddy 1972a, Caddy 1972b, Posgay 1979, Serchuk et al. 1979, Sinclair et al. 1985). Posgay (1979) and Serchuk et al. (1979) estimated that maximum YPR is attained by harvesting 8-year-old scallops. Serchuk et al. (1979), however, suggest that scallops be harvested when they reach age 6, since delaying harvest past this age results in only minor additional increases in YPR. Previous studies have shown that an increase in age at first capture from age 5 to age 6 will result in an increase in YPR of 10% to 20% (Posgay 1962, Serchuk et al. 1979; Table 7). Delaying harvest from age 4 to age 5 will increase YPR by as much as 50% (Sinclair et al. 1985). By the early 1990s, age at first capture had decreased to between ages 3 and 4. It is, therefore, important to investigate the changes in yield that could be expected by delaying harvest to age 4 or older.

TABLE 5.

Estimates of yield (kilograms) over a 2-y time period using the 82.6- and 88.9-mm ring dredges.

Time Period	82.6-mm Rings	88.9-mm Rings
0–6 mo	2,236.53	1,463.82
6–12 mo	1,524.88	1,546.34
12–18 mo	984.35	1,639.49
18–24 mo	634.99	1,178.42
Total	5,380.75	5,828.07

Assuming knife-edge selectivity and no changes in commercial culling size and shucking capacity, delaying harvest from age 3 to age 4 would increase yield in the fishery by nearly 75%. These conclusions are consistent with those presented in other studies that examined YPR for similar sized scallops (Table 7). The offshore scallop dredge, however, does not perform with knife-edge selectivity, and partial recruitment results in the capture and shucking of small scallops. When partial recruitment is considered in the analysis, and fishing mortality of $F = 1.5$ and natural mortality of $M = 0.1$ are assumed, the 88.9-mm ring dredge would increase yield no more than 10% (Table 5).

Our results indicate that advances in yield made possible by gear changes have to be optimized with additional measures to reduce fishing mortality and effort. Posgay (1979) concluded that a cull size of age 5 (95 mm) was too young and the fishing mortality ($F = 0.7$) was too high to achieve maximum yield for the Georges Bank scallop resource during the 1960s. Currently, in the mid-Atlantic fishery, age at recruitment is younger and fishing mortality is higher than on Georges Bank in the 1960s. Increasing ring size to 88.9 mm can increase the yield in the fishery, but additional measures to decrease fishing mortality and effort must be implemented in order to attain the maximum YPR.

Spawning Potential and Spawning Stock Biomass

Data from this study show the larger ring dredge has the potential to improve scallop SSB, which has been depressed due to high exploitation rates, drastically reducing the number of age 4 and 5 scallops. Scallops are sexually mature by the end of their third year (NEFMC 1993), and fall spawning generally occurs between late August and December (Posgay & Norman 1958, MacDonald & Thompson 1986, DuPaul et al. 1989, Schmitzer 1990). It is during this period that the faster growing 3-year-old scallops begin to recruit to the 76.2-mm gear. Most scallops spawn at this time, but the 3-year-old scallops do not contribute significantly to the overall fecundity of the resource (McGarvey et al. 1993). Even considering partial recruitment, the larger dredge rings will decrease harvest of age 3 scallops and allow more scallops to spawn at age 4. Because fecundity of sea scallops increases exponentially with size for several years after first reaching sexual maturity (MacDonald & Thompson 1985, Langton et al. 1987, Carnegie 1994), the delay in harvest should increase the overall fecundity of the resource (Table 6). In addition, McGarvey et al.

TABLE 6.

Estimates of residual reproductive value (RRV) assuming 10% natural mortality and 66% harvest efficiency between ages 3 and 4.

Reference	Study Area	Age (y)	RRV (millions of eggs)	Cumulative Fecundity (millions of eggs)
MacDonald 1984	Sunnyside, Newfoundland	3	4.95	4.95
		4	2.59	7.54
Langton et al. 1987	Boothbay, Maine	3	9.30	9.30
		4	3.96	13.26
MacDonald and Thompson 1986	New Jersey	3	5.49	5.49
		4	3.24	8.73

(1993) found a statistically significant relationship between the number of spawners and recruits for Georges Bank scallop stocks. Delaying harvest by using the larger rings could therefore lead to increases in overall stock abundance and concomitant increases in SSB.

Conclusions

The effects of increasing dredge ring size are strongly dependent on the amount of fishing effort in the study area. A decrease in fishing effort would increase the benefits of the larger ring size, while increasing effort would reduce the positive effects of the larger rings. Analysis conducted for this study suggest that the 88.9-mm dredge rings should provide many benefits to the sea scallop fishery and resource, and combined with the other regulations defined in Amendment 4, should have helped achieve several of the objectives outlined in the FMP. The benefits of the larger ring size suggested by this study have not been realized, however. This is most likely due initially to management measures for other species, and subsequently, similar measures for scallops.

In December 1994, the use of mobile fishing gear, including sea scallop dredges, was prohibited in certain areas of Georges Bank (Closed Area I and Closed Area II) and southern New England (Nantucket Lightship Closed Area) in order to allow severely depleted groundfish stocks to rebuild (NEFSC 1997). Much of the fishing effort previously targeting scallops on Georges Bank was displaced to the southern New England and mid-Atlantic regions, which remained open. Fishing effort substantially increased in these regions (NEFSC 1997). In April 1998, at the request of the New England Fishery Management Council, the Secretary of Commerce closed two additional areas in the mid-Atlantic to scallop fishing to protect small scallops. Recently, the NEFMC (1997) determined that fishing mortality on scallops is still above the overfishing definition, and the closure of these 1,900 square miles in the mid-Atlantic only concentrates fishing effort into the remaining open areas.

Modifications to scallop fishing gear designed to reduce the harvest of small scallops and advance the age of entry can be an important management tool, but are most effective when used in conjunction with measures to reduce or stabilize fishing effort and mortality. Data from this study suggest that increasing scallop dredge ring size can decrease mortality of small scallops and postpone recruitment of scallops to the commercial fishery. The delay in recruitment can lead to increases in fishery yield and spawning potential of the resource. The potential benefits of the recent gear modifications, however, have been diminished due to increased fishing effort in the area. In order to realize the full benefits of increasing scallop dredge ring size, the gear restrictions must be associated with measures to stabilize or decrease fishing mortality and fishing effort.

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TABLE 7.

Results of previous sea scallop yield-per-recruit (YPR) studies.

Reference	Study Area	Age (size) at First Capture	Harvest Delayed Until	Percent Increase in YPR
Posgay 1962	Georges Bank	5	6	18
Serchuk et al. 1979	Georges Bank	5	6	11
Serchuk et al. 1979	Mid-Atlantic	5	6	15
Sinclair et al. 1985	Georges Bank	4	5	55
Serchuk et al. 1979	Georges Bank	(76 mm)	(98 mm)	37
Serchuk et al. 1979	Mid-Atlantic	(77 mm)	(97 mm)	39
Caddy 1972a	Georges Bank	(73 mm)	(92 mm)	65
Present study	Mid-Atlantic	3+ (72.5 mm)	4+ (100 mm)	74

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EFFECTS OF DEPLOYMENT TIME AND ACCLIMATION ON SURVIVAL AND GROWTH OF HATCHERY-REARED SCALLOP (*PECTEN MAXIMUS*) SPAT TRANSFERRED TO THE SEA

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ABSTRACT Hatchery-produced great scallop (*Pecten maximus*) spat of 1 to 5 mm shell height were transferred to a sea-based nursery from March to August 1995. Because the growth season in Norwegian waters is limited by low sea temperature in the spring (5°C–10°C), acclimation to a colder temperature (10°C) than the 15°C in the hatchery was considered in order to enhance survival after deployment. Survival and growth of spat deployed directly to the sea were compared with spat acclimated for 1 and 3 wk. Results indicated that specific growth rates were 1% to 3% per day. Mean survival of acclimated groups was 0% to 9% for small spat (0.7–2.3 mm) and 25% to 36% for bigger spat (4 mm). Acclimation improved mean survival by up to 7% of the small spat and 11% of the bigger spat. An extension from 1 to 3 wk acclimation did not further improve survival or growth. Duration of exposure to low temperatures (<10°C) and temperature at deployment time were main factors affecting survival and growth considering all spat groups, while size at deployment mainly determined the success for acclimated scallops. Less than 5% of the smallest spat survived when directly transferred to sea temperatures of 5°C to 7°C. Larger spat obtained 25% mean survival, which is comparable with 24% to 60% survival of spat transferred to sea temperatures ≥10°C. Shell growth rate likewise was elevated when the temperature exceeded 10°C. Acclimation to a lower temperature gave only limited increase in survival, and it was determined that small spat (<2.6 mm) should not be transferred to temperatures <7°C. By deploying bigger spat (>4 mm) to low sea temperatures, high survival can be expected, and thereby the production period is extended.

KEY WORDS: acclimation, nursery culture, *Pecten maximus*, scallop spat, survival, growth

INTRODUCTION

Stable spat supply to growers is the main constraint in the development of a scallop cultivation industry. Due to overfishing of scallop populations around the world, and to the fact that scallops are high-value products, the establishment of hatcheries for ensuring a reliable production of spat has become important to the industry. Intensive larval growth in a hatchery is usually followed by growth to commercial-sized spat in semi-intensive growth systems termed "nurseries." A nursery can be sea- or land-based, depending on natural seawater supply or cultured algae as a food source. In cold water areas like the North Atlantic, the growth season in the sea is limited by environmental factors such as the low temperature in the spring. The longer the period of the year in which scallops can take advantage of the natural food production, the more beneficial it is to producers. Making spat available earlier in the year and providing growers with larger spat at the optimum time in the growth season would ease the constraint.

Scallops are shown to be susceptible to small changes in temperature (Dickie & Medcof 1963, Strand et al. 1993, Chauvaud et al. 1998), but their tolerance limits varies with species and natural habitat (Brand 1991). Scallops are more sensitive to environmental changes compared with intertidal bivalves like mussels, oysters, and clams because they are not able to completely close their valves when encountering unfavorable conditions. Changes in temperature may have a direct or indirect impact on survival. Temperature drops of 4°C to 7°C have been shown to cause immobility of sea scallops, which in their natural environment could increase vulnerability to predators and thereby increase mortality (Dickie 1958). Abrupt and considerable changes in environmental conditions are likely to occur in an aquaculture situation during handling and transfer operations. The transfer from hatchery conditions to colder temperature conditions in the sea for nursery growth may therefore be hazardous.

In culture, both the thermal and the nutritional conditions will affect scallop growth performance (Ventilla 1982, Wallace & Reinsnes 1985, Andersen & Naas 1993, Couturier et al. 1995, Pilditch & Grant 1999, Grecian et al. 2000). Exposure to cold water temperatures of <10°C have been shown critical for the great scallop, *Pecten maximus*, at different stages. High mortality of veliger larvae occurs at 7°C to 8°C, and at 5°C, total mortality may be expected (Davenport et al. 1975, Beaumont & Budd 1982). For *P. maximum* juveniles of 20 to 30 mm shell height (SH), a reduction in filtration rate of 50% to 80% was recorded at 5°C compared with 9°C (Strand et al. 1993), and Laing (2000) found no growth or measurable uptake of food at 4.7°C by 5 to 14 mm spat. A 100% mortality at <4°C is reported from growth trials in suspended intermediate culture in Norway (Brynjelsen & Strand 1996), while a temperature of 9°C is suggested as a "biological zero" for shell growth in Irish waters (Wilson 1987).

Acclimation may be a successful way to adjust an animal to different conditions. A three-phase process consisting of an immediate response, a stabilization of this response, and a new steady state can explain the adaptation to new surroundings (Kinne 1963). Studies of the sea scallop have indicated that a rise or fall in acclimation temperature of about 5°C may result in a corresponding change in lethal temperature of 1°C (Dickie 1958). The rate of acclimation tends to follow the rate of metabolism, resulting in longer time needed for acclimation to a decrease in temperature than an increase. The animals' environmental history, genetic background, physiological condition, metabolism, age, and size are all factors affecting the capacity, rate, and effect of thermal acclimation (Kinne 1963, Schmidt-Nielsen 1990).

One method for producing *P. maximus* spat in Norway is intensive hatchery rearing to approximately 2 mm SH at a temperature of 15°C, before further growth to 15 mm in a sea-based nursery. Transfer to the sea is restricted to the period from June to August for the spat to reach a commercial size within one season. A prolonging of the production period would be possible if a method was found to successfully transfer the spat to the sea earlier in the spring when the temperature is low. The prevailing method of transferring spat from the hatchery is by directly deploying the spat to the sea. Acclimation of spat to an environment of 10°C with subsequent transfer to temperatures between 5°C and

10°C was considered a possible solution to ensure that scallop spat would tolerate transfer to the sea during the spring.

The aim of this study was to determine whether exposing the spat to a temperature between the hatchery and the sea temperature, before transfer to the sea in the spring, could enhance survival. Spat groups that had been subjected to the prevailing production method of directly deployment to the sea were monitored throughout the season, and survival and growth rates were compared with spat groups acclimated for 1 and 3 wk.

MATERIALS AND METHODS

The study was carried out during spring and summer 1995 in Oygarden, Hordaland County, western Norway, with spat originating from broodstock collected from the wild. During the period from October 1994 to June 1995, groups of 40 to 60 scallops were conditioned, induced to spawn, and cross-fertilized. Spat of 1 to 5 mm SH were obtained by standard production methods at Oygarden Scallop Hatchery. Survival and growth of spat from 10 different spawning groups (1–10) were determined after deployment to the sea throughout the production season. Spat from spawning groups 3 through 6 were subjected to acclimation treatments for 1 and 3 wk before transfer to the sea (Table 1). For some of the spawning groups, more spat groups of different age and size (A, B, and C) were deployed (Table 1).

Prior to deployment, the spat were removed from 140-μm mesh screens, which served as bottom of cylindrical growth containers or sieves (1,225 cm^2), by gentle brushing in a flow of water, and they were relayed onto plastic trays (60 × 60 × 8 cm) covered with 500 μm mesh. The trays were kept overnight in running seawater, allowing resettlement of the spat. Stacks of four trays, consisting of three experimental trays plus one as lid, were suspended from long lines in the sea at a depth of 8 to 10 m. Grow-out in the sea was within the period from March 1 to September 29 (Table 1). The growth time varied according to deployment date, as the spat grew to a size of 5 to 10 mm, big enough for grading and restocking.

Acclimation took place under conditions of lower temperature and food concentration than were used prior to the treatment. The spat were kept at 10°C and were fed 10 cells per μl of a mix of the monocultured algae *Pavlova lutheri*, *Isochrysis galbana*, and *Skeletonema costatum* in a 1:1:2 ratio, compared with 15°C and 15 to 20 cells per μl, respectively. The spat groups (3, 4A, 4B, 5, and 6A) subjected to acclimation were divided into three subgroups, each consisting of three sieves. One subgroup was deployed directly to the sea without acclimation. The second subgroup was deployed after 1 wk of exposure to acclimation conditions, and the third was transferred after 3 wk of acclimation. Because of limitations in the hatchery, the spat were transferred directly from hatchery conditions to acclimation conditions without a gradual habituation to the lower temperature.

Survival was estimated as the difference in numbers of animals at deployment time and the numbers retained on a 3-mm screen after 52 to 134 days grow-out in the sea. The initial number in each sieve was estimated by counting the spat of a 50 × 1 cm^2 (4%) area of the mesh bottom and multiplying to the total area. The final numbers were estimated by wet weight measurements based on counting subsamples of 100 spat from the trays.

TABLE 1.

Age, size, density, temperature, and grow-out time in the sea of cultivated scallop spat deployed directly to the sea from the hatchery (0), or transferred after 1 or 3 wk acclimation to a temperature of 10°C.

Spat Group	Acclimation (wk)	Trays Deployed (no.)	Deployment Date in 1995	Age at Deployment (days)	Size at Deployment (mm)	Density at Deployment (no. cm^{-2})	Sea Temperature at Deployment (°C)	Grow-Out Time (days)
1	0	3	Mar 01	131	2.6	2.8	5.9	133
2	0	3	Mar 01	106	2.6	3.1	5.9	134
3	0	3	Mar 01	86	2.3	3.0	5.9	133
	1	3	Mar 06	91	2.2	2.8	5.7	128
	3	3	Mar 21	106	2.0	3.6	5.1	113
4A	0	3	Mar 21	56	1.1	2.6	5.1	113
	1	3	Mar 29	64	1.3	2.7	5.3	107
	3	3	Apr 11	77	1.7	2.9	5.6	93
5	0	3	Apr 11	56	0.7	2.7	5.6	93
	1	3	Apr 20	65	0.9	2.4	5.5	85
	3	3	May 04	79	1.2	2.1	7.0	71
6A	0	3	Apr 11	56	0.8	2.8	5.6	92
	1	3	Apr 20	65	1.3	2.9	5.5	85
	3	3	May 04	79	1.6	2.9	7.0	71
4B	0	3	Apr 28	94	4.0	3.3	6.2	77
	1	3	May 04	100	4.0	4.0	7.0	71
	3	3	May 18	114	4.3	3.4	7.1	68
6B	0	2	May 31	106	3.6	2.5	10.0	55
4C	0	2	May 31	127	5.6	1.7	10.0	55
6C	0	2	May 31	106	5.0	1.7	10.0	55
7	0	3	Jun 20	63	1.7	1.9	11.5	57
8	0	3	Jun 22	51	1.6	3.2	11.5	55
9	0	3	Aug 01	63	1.9	2.4	12.8	59
10	0	3	Aug 08	56	1.5	2.7	13.0	52

Growth in the sea was based on mean SH measurements of 50 animals from each sieve at deployment time and from each tray at uptake date. Spat growth during the acclimation period was also measured. Final size of the bigger (4B, 4C, 6B, and 6C) deployed spat was determined by using the weighted average of means from two size groups (3–10 mm and >10 mm). SH growth was calculated as specific growth rate (% day^{-1}): $G = (e^{g} - 1) \times 100$, where the instantaneous daily growth coefficient $g = (\ln SH_{final} - \ln SH_{initial})$ per days (Ricker 1979, Claus 1981).

Sea temperature and salinity were measured weekly with a WTW Microprocessor Conductivity Meter (LF196) (Wissenschaftlich-Technische Werkstätten G.M.B.H. D-8120, Weilheim, Germany) connected with a WTW conductivity-measuring cell (TetraCon 96 A-4). Food concentration in the sea was measured occasionally using an electronic particle counter (Model ZM Coulter Counter, Coulter Electronics Limited, Luton, England).

The statistical analyses were carried out using STATISTICA®, version 5, and a significance level of 0.05 was applied to the tests. Analysis of variance (ANOVA) was performed on the growth and survival data of the spat that were acclimated, and treatments showing significant differences were further characterized by the Tukey HSD test. The survival data (percentage) of the spat were arcsine transformed before statistical analysis to obtain variance homogeneity for all groups (Sokal & Rohlf 1995). Pearson product-moment correlation coefficients were calculated for shell parameters and grow-out conditions in the sea. Multiple forward stepwise regression analysis was performed for all the spat groups transferred to the sea and for acclimated groups separately. Survival (percentage) and specific growth (percentage per day) were related to the variables; acclimation time, size, density, and sea temperature at deployment time, and the period of exposure to temperatures <10°C during grow-out in the sea.

RESULTS

Seawater Conditions

Sea temperature at the grow-out depth of 8 m increased from 5°C to 15°C during the experimental growth period (Fig. 1). The temperature reached 7°C in the beginning of May and exceeded 10°C in June. Salinity varied between 30 and 33 with occasional drops below 30‰. A salinity of 27.6 was the lowest recorded during the period from June to October (Fig. 1). The number of 2- to 10-μm particles at 8 m was below 10 cells per μl in March and April. In the beginning of July, the natural food production had increased, and 50 cells per μl was measured.

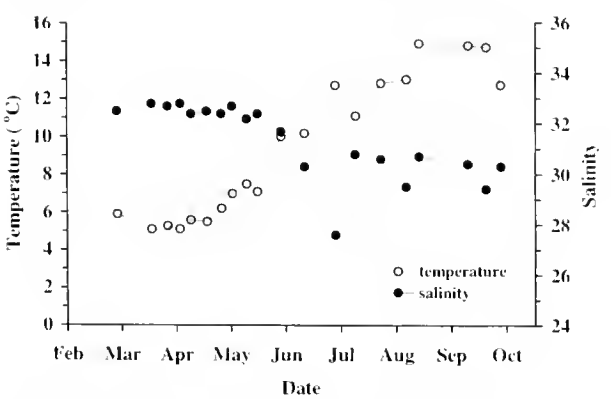

Figure 1. Temperature and salinity at 8 m grow-out depth during the 1995 season, Ulvesundet, Oygarden, Norway.

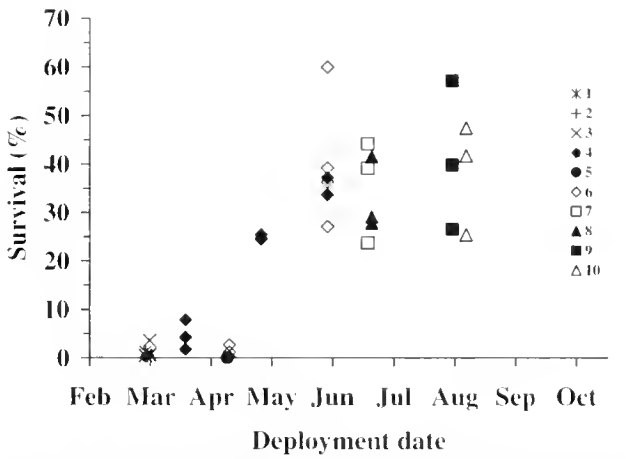

Figure 2. Survival of scallop spat directly deployed to the sea during the 1995 season. Different symbols represent the spat groups number 1 through 10, and each point represents the survival in one tray.

Survival

Survival ranging from 0% to 60% was observed for scallop spat transferred directly from the hatchery to the sea during the 1995 production season (Fig. 2). Dead scallops found upon retrieval passed through the 3-mm screen, indicating that no growth had taken place before mortality occurred. Spat in the size range 0.7 to 2.6 mm directly deployed to a sea temperature below 7°C showed mean survival of less than 5%, while the 4B group, which held a 4 mm SH at deployment, showed 25% survival (Fig. 2; Table 1). A large variation in survival between trays was shown for the spat groups deployed at a sea temperature of 10°C and above. The average survival was 38% (SD = 10.37) for these groups, ranging from 24% to 60% (Fig. 2).

The spat groups (3, 4A, 4B, 5, and 6A) subjected to the acclimation treatment were deployed from March 1 to May 18 when the sea temperature ranged from 5°C to 7°C (Table 1). Mean survival rates from 0% to 9% were obtained for the spat groups of initial average size 0.7 to 2.3 mm. For the 4-mm spat group (4B), mean survival rates of 25% to 36% were found (Fig. 3). Compared to

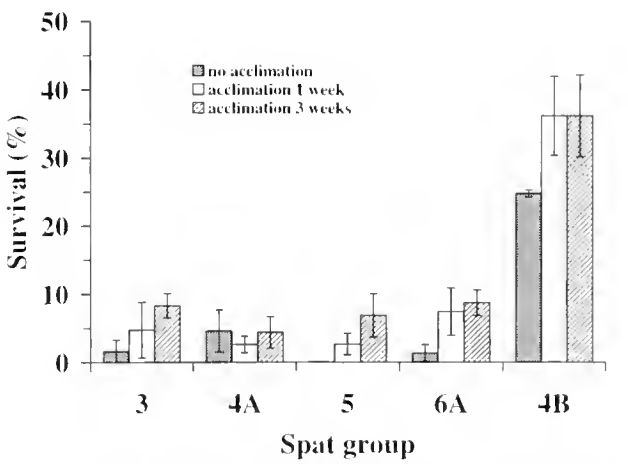

Figure 3. Mean survival of scallop spat subjected to acclimation treatments. Subgroups of spat were deployed directly to the sea (no acclimation) and after 1 and 3 wk of acclimation. Vertical bars show standard deviation.

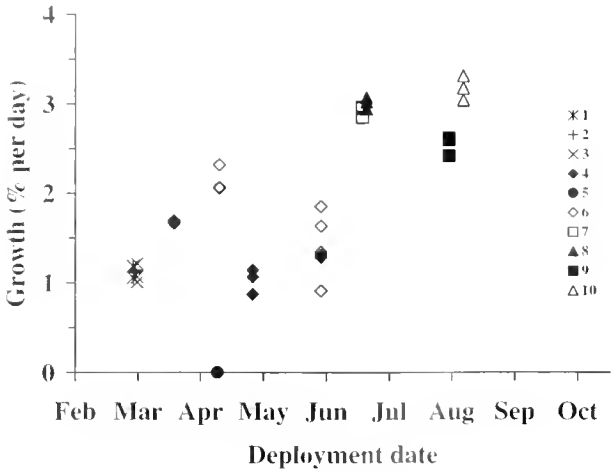

Figure 4. Specific growth rate (percentage of SH per day) of scallop spat directly deployed to the sea during the 1995 season. Different symbols represent the spat groups number 1 through 10, and each point represents the mean growth in one tray.

direct transfer from the hatchery to the sea, acclimation improved the mean survival by up to 7% for the smaller sized spat groups, while the bigger sized group gained a mean increase of 11% (Fig. 3). A one-way ANOVA was carried out for each spat group. The survival of the acclimated spat from the groups number 3 and 4A was not significantly different from the spat deployed directly to sea. For the other spat groups examined, there was a significant difference between the treatments. Survival of the spat transferred directly to the sea was significantly lower than the survival of acclimated spat. Extension of the acclimation time from 1 to 3 wk did not improve survival significantly for any of the spat groups.

Growth

Specific growth rates of the spat deployed to the sea without foregoing acclimation were found to be in the order of 0.9% to 3.3% or 46 to 128 μm increase in SH per day (Fig. 4). No surviving scallops were observed for spat group 5 (Fig. 2), which consisted of the smallest spat deployed during the experiment (Table 1). Hence, no growth could be calculated for this group.

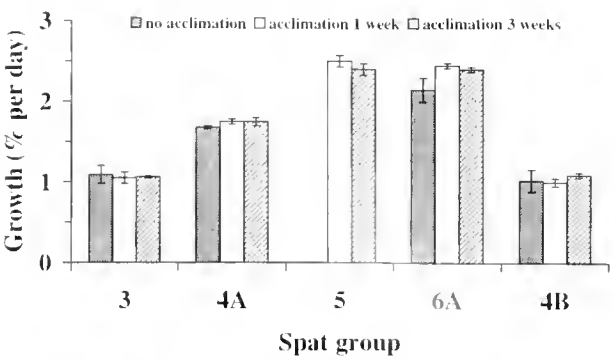

Figure 5. Mean specific growth rate (percentage of SH per day) of scallop spat during the total experimental period (acclimation time included). The spat were deployed directly to the sea (no acclimation) and after 1 and 3 wk of acclimation. Vertical bars show standard deviation.

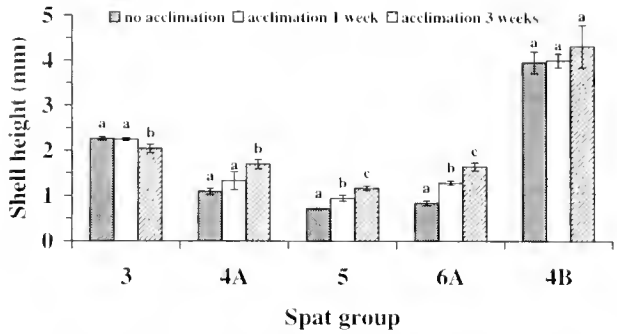

Figure 6. Mean SH at deployment time of scallop spat subjected to acclimation treatments. Subgroups of spat were deployed directly to the sea (no acclimation) and after 1 and 3 wk of acclimation. Values indicated by different letters within each spat group are significantly different. Vertical bars show standard deviation.

The mean final size of the spat groups surviving direct transfer to the sea was 9.1 mm (SD = 1.74). The SHs ranged from 3 to 21 mm.

The mean specific growth rates obtained for the spat groups subjected to acclimation were between 1.0% and 2.5% per day during the grow-out period in the sea, as for the total experimental period, acclimation time included (Fig. 5). The statistical tests showed that acclimation of the spat group 6A significantly improved the growth rate during the total experimental period, and of the spat from group 3 acclimated for 3 wk, considering the grow-out phase in the sea. An extension of the acclimation time from 1 to 3 wk did not result in faster growth for any other spat groups. The SH at time of deployment to the sea differed between the subgroups, and except for group 3 and 4B, the spat groups subjected to acclimation treatment showed growth during the acclimation period (Fig. 6). Upon retrieval, the differences in average SH were evened out, with the exception of the 6A subgroup not acclimated, which obtained a significantly smaller size compared with the acclimated ones (Fig. 7).

Correlation and Regression Analyses

Growth rate and survival are related to short grow-out time at higher temperatures (Table 2). Growth rate correlated with small spat, and survival correlated with large spat. Some of the shell and

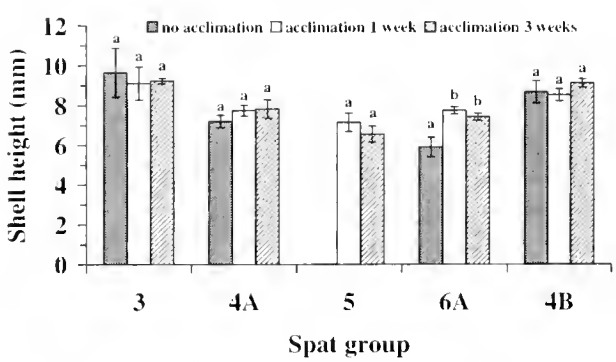

Figure 7. Mean shell height upon retrieval of scallop spat subjected to acclimation treatments. Subgroups of spat were deployed directly to the sea (no acclimation) and after 1 and 3 wk of acclimation. Values indicated by different letters within each spat group are significantly different. Vertical bars show standard deviation.

TABLE 2.

Pearson product-moment correlation matrix for specific growth (% day^{-1}), % survival (transformed), acclimation time, age (days from spawning), size (shell height), density (no. cm^{-2}), and sea temperature at deployment, average temperature during grow-out, total grow-out time in the sea, and time exposed to sea temperatures <10° C. Significant correlation coefficients are indicated with asterisks (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$).

Variable	Growth	Survival	Acclimation	Age	Size	Density	Deployment Temperature	Average Temperature	Grow-Out Time
Survival	0.34**								
Acclimation	0.00	−0.09							
Age	−0.54***	0.10	0.17						
Size	−0.40**	0.55***	−0.05	0.77***					
Density	−0.26*	−0.11	0.22	0.15	0.05				
Deployment temperature	0.59***	0.77***	−0.33*	−0.18	0.19	−0.32*			
Average temperature	0.63***	0.80***	−0.20	−0.27*	0.13	−0.26	0.96***		
Grow-out time	−0.51***	−0.79***	−0.02	0.23	−0.23	0.24	−0.74***	−0.85***	
Grow-out <10° C	−0.52***	−0.82***	0.02	0.20	−0.26*	0.27*	−0.79***	−0.88***	0.99***

environmental parameters correlated strongly between themselves, like size and age at deployment, temperature at deployment and during the grow-out period, and grow-out time and temperatures (Table 2). According to the stepwise regression analysis, exposure time to temperatures <10° C and size at deployment explained 80% and 90% of the variation in survival of all groups and acclimated groups, respectively (Table 3). Temperature at deployment ranked above the other variables in explaining the growth rate variation regarding all spat groups during grow-out in the sea. Size was the most important factor for the spat acclimated 1 and 3 wk before

TABLE 3.

Multiple forward stepwise regression analysis between % survival (transformed) and specific growth rate (% day^{-1}) in the sea, and the variables acclimation time, size, density, sea temperature at deployment, and the duration of exposure to temperatures <10° C in the sea. The analysis was applied to all the spat groups deployed and for groups acclimated 1 and 3 wk before transfer.

	Stepwise Results							Multiple Results			
									Analysis of Variance		
Dependent Variable/ Independent Variable	Step	Multiple R^2	Partial Correction	F Enter	B	t Value	P Level	Intercept	df	F	P Level
Survival											
all groups											
Time <10° C	1	0.674	−0.51	138.393	−0.186	−4.679	0.0000	−5.864	5, 63	74.344	0.0000
Size	2	0.796	0.67	39.749	0.004	7.174	0.0000				
Temperature	3	0.842	0.51	18.792	2.374	4.666	0.0000				
Density	4	0.852	0.24	4.481	0.001	1.922	0.0592				
Acclimation	5	0.855	0.13	1.163	0.732	1.078	0.2850				
acclimated groups											
Size	1	0.811	0.82	119.892	0.007	6.988	0.0000	6.735	5, 24	44.497	0.0000
Time <10° C	2	0.899	−0.54	23.464	−0.155	−3.125	0.0046				
Density	3	0.901	0.17	0.731	0.000	0.831	0.4141				
Acclimation	4	0.903	−0.11	0.279	−0.377	−0.534	0.5983				
Temperature	5	0.903	0.03	0.018	0.212	−0.132	0.8957				
Growth											
all groups											
Temperature	1	0.349	0.48	35.921	0.182	4.368	0.0000	1.308	5, 63	26.301	0.0000
Size	2	0.620	−0.68	47.055	−0.000	−7.292	0.0000				
Acclimation	3	0.665	0.26	8.747	0.117	2.103	0.0395				
Time <10° C	4	0.675	−0.17	2.025	−0.004	−1.361	0.1783				
Density	5	0.676	−0.05	0.154	−0.000	−0.392	0.6961				
acclimated groups											
Size	1	0.630	−0.96	47.739	−0.000	−16.704	0.0000	3.233	5, 24	153.271	0.0000
Time <10° C	2	0.964	−0.90	249.489	−0.014	−9.848	0.0000				
Density	3	0.967	−0.24	2.378	−0.000	−1.237	0.2281				
Acclimation	4	0.969	−0.26	1.350	−0.027	−1.321	0.1990				
Temperature	5	0.970	0.18	0.803	0.041	0.896	0.3790				

deployment. Density neither had significant effect on survival nor on growth, and acclimation time had no significant influence within the acclimated groups (Table 3).

DISCUSSION

The present study shows that the success of scallop spat is highly dependent on temperature at deployment time. The results indicate that transfer of small *P. maximus* from hatchery to natural sea conditions of temperatures <7°C for long durations is likely to be critical. Survival of the spat deployed directly to the sea increased substantially when the sea temperatures reached 7°C (Figs. 1 and 2). There was a strong positive correlation between survival and temperature, despite the wide variation between trays (Fig. 2; Table 2). Lethal temperatures for *P. maximus* larvae and juveniles, according to other studies, are 8°C and <4°C, respectively (Beaumont & Budd 1982, Brynjelsen & Strand 1996). The spat investigated in our study were sized between larvae and juveniles. On the basis of this, it was assumed that transfer of spat to sea temperatures >5°C would not be fatal. Our observations show that some *P. maximus* spat of <2.5 mm are able to survive transfer to 5°C to 7°C, while others suffer high mortality. The low lethal tolerance temperature for another cold water scallop species, *Patinopecten yessoensis*, is also found to be about 5°C (Ventilla 1982), while the sea scallop, *Placopecten magellanicus*, tolerate temperatures down to below 0°C (Couturier et al. 1995). The lower lethal temperature of scallop spat might not be an absolute temperature but rather a range. Each scallop species is distributed within a certain geographical and bathymetric range where the environmental conditions support survival (Brand 1991). Tolerance limits, therefore, will vary between populations of the same species due to the exposure of different local temperature ranges and seasonal variations. Within a population, a high temperature shown to be lethal to the animal during winter may be tolerated by the animal when exposed to summer conditions and vice versa (Schmidt-Nielsen 1990).

A marked increase in shell growth rates was shown at temperatures above 10°C (Fig. 4; Table 1). Several studies have concluded that temperature rather than food availability is the most important environmental factor affecting scallop growth in the sea (Andersen & Naas 1993, Kleinman et al. 1996, Chauvaud et al. 1998, Laing 2000). Others (MacDonald & Thompson 1985, Wallace & Reinsnes 1985, Thorarinsdóttir 1994, Laing & Psimopoulous 1998) stress the importance of food supply for scallop growth. Which of the two factors are most important could not be determined in our study due to lack of regular food concentration measurements in the sea. The food rations in terms of particle counts were lower from March to May compared with later in the season, but the level was not believed to be growth limiting. Spring bloom events of algae probably ensured enough food since similar growth rates of surviving spat were found both early and late in the season.

The low survival in spring found in our study may also be influenced by environmental factors other than temperature and food availability. More site-specific conditions like salinity, flow velocity, presence of predators, and fouling organisms are shown to affect growth and survival of scallop juveniles (Wilson 1987, Cahalan et al. 1989, Wildish & Saulnier 1992, Andersen & Naas 1993, Lodeiros & Himmelman 1996, Freites et al. 2000, Grecian et al. 2000). Likewise will the different environmental factors synergistically affect the growth performance (Kirby-Smith & Barber 1974, Paul 1980, Strand et al. 1993, Grant 1996, O'Connor & Heasman 1998, Pilditch & Grant 1999). In suspended culture, the scallops are exposed to more fluctuations in food supply, salinity, and temperature compared with their natural habitat on the sea bottom (Tebble 1966). By keeping the animals higher up in the water column, as in our study, the temperature and food amount are elevated during spring and summer. The higher survival and growth observed for spat exposed to summer temperatures was probably a result of increased metabolism together with food available in excess. Oxygen consumption and filtration rate of scallops are known to be temperature dependent (McLusky 1973, Bricelj & Shumway 1991), as are energy metabolism, feeding ability, and growth of bivalves in general (Walne 1972, Bayne et al. 1976, de Villiers et al. 1989, Rice & Pechenik 1992, Grant 1996).

Relatively high growth rates were obtained for the spat deployed in the spring (Fig. 4). The growth was based on measurements of spat that had survived the total 7 to 19 wk grow-out in the sea. Seen together with the complementary low survival of the spat transferred early in the year, the good results are due to a few, but very vital, scallops. The growth rate calculations were based on the total grow-out period in the sea. A longer time span combined with a lower average temperature was expected to give a lower daily growth compared with animals allowed to grow at optimal time in the sea. This was confirmed by the significant negative correlation coefficients found between grow-out time and growth (Table 2).

Acclimation from 15°C to 10°C reduced the negative impact of cold water temperature at deployment. According to the regression analysis, size was the most important factor explaining the variation in survival and growth of acclimated *P. maximus* spat (Table 3). The acclimated spat were transferred to the similar temperature range (5.1°C–7.1°C), which might account for the reduced effect of temperature at deployment. Larger spat (4 mm) showed better survival than smaller spat (<2.6 mm) when transferred to low temperatures. Along with the size at deployment, a shorter time of exposure to temperatures below 10°C might have influenced the results, as this was also shown as an important factor explaining growth and survival variation. Grecian et al. (2000) likewise found initial size to have significant influence on growth and survival of *P. magellanicus* spat transferred to sea-based nursery, showing better results for spat of >3 mm SH compared with spat of 1.2 to 2 mm. For *Pinctada margaritifera* spat, on the other hand, it was found that transfer from the hatchery to the sea should occur as soon as possible after settlement in order to maximize growth (Pit & Southgate 2000).

The scallops found dead upon retrieval in the present work were of a small size (<3 mm SH) compared with the majority of the surviving ones. Mortality is therefore likely to have occurred shortly after deployment time. According to the descriptions of the gill organogenesis by Beninger et al. (1994), the spat in our study were anatomically undeveloped. Beninger et al. (1994) found the gill function of *P. maximus* spat up to 4 mm to be different from the adult gill, and suggested that the development from one size stage to the next is critical in terms of survival. This may explain that the spat of 0.7 to 2.6 mm SH used in our study were incapable of rapid adjustment after transfer to the cold water environment. Poorer feeding ability of the spat deployed to the lowest temperatures in addition to the temperature stress may have caused the mass mortalities observed in spring.

The acclimation to an intermediate temperature between the hatchery (15°C) and the sea (5°C–7°C) prior to deployment in spring enhanced the survival of scallop spat. The present study shows increased mean survival of up to 7% for small spat (0.7–2.3

mm) and 11% for larger spat (4 mm). The overall survival rates of the larger spat were higher and comparable to those obtained for spat deployed later in the season (Figs. 2 and 3). Regarding growth rates of the larger spat, they were relatively low, and no improvement was gained from acclimation (Figs. 4 and 5). These results could be due to the fact that smaller individuals of scallops grow faster than larger ones (Bricelj & Shumway 1991, Parsons et al. 1993) (Table 2), or that growth in sea conditions, in spite of suboptimal temperature, is more favorable than hatchery conditions. With respect to the SH of the spat subjected to acclimation, some growth compensation seems to have occurred in the sea since similar final sizes were achieved for the subgroups (Figs. 6 and 7). The spat of group 5 and 6A not acclimated and directly deployed to a temperature of 5.6°C appeared, on the other hand, not to reveal such growth compensation. One group suffered total mortality and the other showed a significantly lower final SH compared with the acclimated spat subgroups. These spat, which were 0.7 and 0.8 mm SH at deployment, belonged to the gill development stage 2 described by Beninger et al. (1994). The other spat in the study were stage 3 at transfer, further implying that the spat were transferred to the cold seawater conditions at a critical stage in life.

Since no significant difference in survival and final SH was found between the groups acclimated for 1 and 3 wk, we believe that 1 wk is sufficient for small scallop spat to adapt to 10°C. Adaptation of mussels to a change in temperature is shown by an immediate response in oxygen consumption and filtration rate, followed by a 2-wk acclimation period for physiological compensation to initial level (Bayne et al. 1976). The observed growth during the acclimation period for most groups (Fig. 6) confirms that 1 to 4 mm *P. maximus* spat can tolerate an abrupt change from 15°C to 10°C. Exposure to a decrease in temperature from 15°C to 5°C to 7°C, on the other hand, seemed to be too stressful for the small spat (0.7–2.6 mm). Compared with the survival (24%–60%) obtained for spat transferred to sea temperatures more equal to the hatchery temperature, the mean survival was low (<9%). A gradual exposure to cold water before deployment early in the season might be a better solution for adjusting the temperature tolerance limit for scallop spat. Laing (2000) successfully acclimated spat of 5 to 14 mm from a rearing temperature of 17°C to 5°C by temperature reduction rates of no more than 1°C per day. The constant and fluctuation temperature regime studied by Pilditch and Grant (1999) did not affect the shell growth rate of *P. magellanicus* differently, but limited ability to alter metabolic energy demands following temperature changes was shown. It is possible that *P. maximus* also has limited capability to regulate its metabolism to sudden changes of low temperatures. Thus, the success of spat production is highly dependent on deployment time, which was also shown for *P. magellanicus* (Grecian et al. 2000), and growth performance will depend on initial scallop vitality before abrupt transfer to colder water.

Temperature at deployment and the period of exposure to low temperatures are found to be the main factors affecting survival and growth for small *P. maximus* spat (1–5 mm) transferred from hatchery to sea conditions. Improvement of tolerance to cold water environment is shown by acclimation to lower temperature, leaving size at transfer a critical factor. From an economical point of view, the result was of limited interest, while the maximum mean survival did not exceed 9% for the smallest acclimated spat. To ensure high survival rates, spat of <2.6 mm SH should not be transferred to sea temperatures below 7°C. Alternatively, enhanced survival of spat transferred to temperatures from 5°C to 7°C may be achieved by deploying bigger spat (>4 mm). If the spat could be kept in the hatchery to approximately 4 mm and acclimated to a colder temperature, we believe that an earlier start of the growth season in early spring is possible.

ACKNOWLEDGMENTS

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HETEROZYGOTE DEFICIENCIES AND GENOTYPE-DEPENDENT SPAWNING TIME IN *MYTILUS EDULIS*

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ABSTRACT Deficiencies of heterozygotes against the expectations of the Hardy-Weinberg model are commonly observed in wild populations of marine bivalves and genotype-dependent spawning time has been proposed as a possible cause of heterozygote deficiency. Adult mussels, *Mytilus edulis* L., were collected every fortnight at two locations in the Menai Strait, North Wales during the spawning season in 1994 and induced to spawn artificially. Genetic analysis at ten allozyme loci revealed no significant differences in allele frequencies or heterozygosity between spawners and non-spawners during the season. It was concluded that the time that individual mussels spawned was not dependent on their genotype at these ten loci and could therefore not be an explanation for the significant heterozygote deficiencies observed at several loci in this study. The presence of null alleles and/or selection against heterozygotes was suggested as the most likely causes of heterozygote deficiency.

KEY WORDS: allozymes, heterozygote deficiency, *Mytilus edulis*, spawning time

INTRODUCTION

Deficiencies of heterozygotes against the expectations of the Hardy-Weinberg model are commonly reported in allozyme studies of bivalves (Gartney-Kepkay et al. 1980, Skibinski et al. 1983, Zouros & Foltz 1984, Gosling & Wilkins 1985, Volckaert & Zouros 1989, Beaumont 1991, Gosling 1992) and several explanations have been offered. The extensive larval dispersal exhibited by bivalves would tend to rule out the Wahlund effect (the result of sampling differentiated sub-populations or mixtures of cryptic species, Raymond et al. 1997) or inbreeding and, although null alleles certainly occur in some populations of bivalves (Gaffney 1994, Hoare & Beaumont 1995), they need to be present at unexpectedly high frequency to explain the observed levels of heterozygote deficiencies. There may be differential selection against heterozygotes during different life stages (Hu et al. 1993). Indeed, Beaumont (1991) has demonstrated the generation of heterozygote deficiencies at the post-larval stage in laboratory-reared mussels, presumably through selection against heterozygotes, but evidence for this is extremely difficult to detect in natural populations. One explanation proposed by Zouros and Foltz (1984) is that spawning within a population of bivalves may be genotype-dependent. If, for example, homozygotes for the common allele at a locus tend to spawn at a different time, say a few days before or a few days after other genotypes in the population, then panmixia is disrupted and a deficiency of heterozygotes will occur at that locus among the offspring. Gosling and Wilkins (1985) have also proposed "that in species which have an extended spawning period, within a single locality, the biochemical genotype of individuals may be important in regulating spawning synchrony". Ríos et al. (1996) record apparent differences in spawning time between individuals with different heterozygosities in a Mediterranean population of *Pecten jacobaeus* L. but the relationship between genotype and spawning time has yet to be tested in any other bivalve species.

Temperature and food supply are considered to be the most important factors controlling gametogenesis and spawning in *Mytilus edulis*, although there may be an interaction between these and other exogenous and endogenous factors (Seed & Suchanek 1992). Mussels may spawn following a variety of stimuli (Seed & Suchanek 1992), but only those mussels which have completed gametogenesis and are "ripe" will spawn as a result of an artificial stimulus. The objective of the study presented here was to detect any possible allozyme genetic differences between those *M. edulis* which would spawn and those which would not, throughout the spawning season, using artificial spawning induction as a ripeness indicator.

MATERIALS AND METHODS

Adults of *Mytilus edulis* were collected from a rocky shore at Gallows Point, near Beaumaris (53°15′N, 4°5′W), and by the island of Ynys Faelog, Menai Bridge (53°14′N, 4°9′W), North Wales (referred to as 'Beaumaris' and 'Menai Bridge', respectively). From January to June 1994, every fortnight during low tide, *ca.* 30 mussels were collected from these sites and brought into the School of Ocean Sciences (University of Wales, Bangor) laboratory and held at 6 ± 1°C in tanks with a continuous flow of sea water until spawning induction (Table 1). In mid-April 1994, samples were collected within three days of each other, but the samples from Menai Bridge were lost due to a technical failure in the holding tank. Usually spawning trials were attempted within three days of collection, but when trials were delayed for longer than three days, a mixture of the micro-algae *Pavlova lutheri* (Droop) Green, *Rhinomonas reticulata* (Lucas) Novarino, *Skeletonema costatum* (Greville) Cleve and *Tetraselmis chui* (Kylin) Butcher was drip-fed into the tanks as a food supplement. Trials were always conducted within 15 days of collection. Spawning was induced by the injection of 2 ml of 0.5 M KCl into the mantle cavity of individual mussels that were then left out of water for one hour. Each mussel was then placed into a 250-ml glass jar and covered with 0.2 μm filtered and UV light treated seawater (FSW), and left at 16°C undisturbed for 24 hours. For each mussel in the trial, the maximum linear dimension of the shell was measured to the nearest 0.1 mm and the individual was categorised as a male, a female or a non-spawner.

Samples of posterior adductor muscle and digestive gland were taken from each individual to be genetically analysed, placed together in a microtube and frozen at −75°C, until electrophoresis.

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TABLE 1.

***Mytilus edulis*. Sampling date, sample size (n) and mean length (mm) of mussels collected in Beaumaris and Menai Bridge during the 1994 spawning season.**

Beaumaris					Menai Bridge				
Date		n	mean	se	Date		n	mean	se
28-Jan	nsp	23	63.26	1.74	28-Jan	nsp	28	60.02	0.84
	spa	7	57.71	2.88		spa	3	63.13	3.82
11-Feb	nsp	24	59.90	1.58	11-Feb	nsp	25	59.46	1.18
	spa	6	60.32	2.85		spa	6	60.02	1.75
25-Feb	nsp	24	66.16	1.44	25-Feb	nsp	19	59.52	1.25
	spa	6	59.07	2.60		spa	11	57.68	1.18
12-Mar	nsp	19	64.56	1.38	15-Mar	nsp	15	60.82	1.04
	spa	11	61.93	2.17		spa	15	65.00	1.56
25-Mar	nsp	14	63.03	1.30	30-Mar	nsp	23	58.89	1.06
	spa	25	61.24	1.24		spa	18	56.61	1.52
10-Apr	nsp	19	60.11	1.42					
	spa	17	57.32	1.05					
13-Apr	nsp	15	65.49	1.67					
	spa	15	57.21	1.06					
27-Apr	nsp	12	61.42	2.34	27-Apr	nsp	14	58.81	1.31
	spa	19	64.04	1.58		spa	14	62.65	1.44
12-May	nsp	10	64.83	1.64	12-May	nsp	19	60.49	1.26
	spa	20	64.40	1.68		spa	13	59.96	1.71
27-May	nsp	22	62.53	1.28	27-May	nsp	32	58.58	0.92
	spa	19	61.21	1.66		spa	10	57.54	1.06
12-Jun	nsp	33	62.53	1.42	12-Jun	nsp	33	59.29	1.03

nsp = non-spawner; spa = spawner; se = standard error.

All individuals which spawned (up to a maximum of 13) and about 10 non-spawners from each trial were scored. Starch gel electrophoresis was performed for ten enzymatic systems: NADH-diaphorase (DIA, 1.6.2.2), esterase-D (ESD 3.1.1.1), glucose phosphate isomerase (GPI 5.3.1.9), glutathione reductase (GSR 1.6.4.2), inorganic pyrophosphatase (IPP 3.6.1.1), leucine amino peptidase (LAP 3.4.11.-), mannose-phosphate isomerase (MPI 5.3.1.8), octopine dehydrogenase (ODH 1.5.1.11), 6-phosphogluconic dehydrogenase (PGD 1.1.1.44), phospho- glucomutase (PGM 2.7.5.1). Gels stained for ESD, GPI and LAP were run in TME buffer (0.1 M Tris, 0.1 M maleic acid, 0.01 EDTA and 0.01 $MgCl_2$, pH 7.4). Gels stained for DIA, GSR and IPP were run using a 0.1 M Sodium Citrate pH 7.4 buffer gel and run in TME pH 7.4 electrode buffer. TME buffer at pH 6.0 was used for ODH and PGM gel and electrode buffers (Beaumont & Beveridge 1983, Gentili & Beaumont 1988). MPI was run as described by McDonald et al. (1991) together with PGD. LAP and ODH were stained as described by Beaumont et al. (1983), and Beaumont et al. (1980), respectively. Other enzymes were stained following Harris and Hopkinson's (1976) procedures, except that meldola blue was used instead of PMS (Turner & Hopkinson 1979). Allele nomenclature designated the commonest allele as '100' and the other alleles were given numbers according to their electrophoretic mobility relative to the 100 allele.

To test shell length against spawning time, two-factor ANOVA for mussel length was performed using the sampling date and whether or not the mussel spawned as the two factors analysed (Fry 1993). A few trials early and late in the spawning season produced fewer than four spawners and these trials were excluded from the analysis.

Spawners and non-spawners were considered as separated subpopulations. The data set was analysed using BIOSYS-1 (Swofford & Selander 1981) to calculate allele frequencies and unbiased heterozygosities (Nei 1978). Agreement with the Hardy-Weinberg (HW) model was tested using the exact test (Genepop: Raymond & Rousset 1995) and the direction of any deviation from the model was indicated by the sign of Wright's (1951) fixation index *Fis*. Mean unbiased individual heterozygosities were compared for each sample using the *t*-test (Sokal & Rohlf 1995). Chi-square contingency table analysis was used to determine whether there was any relationship between the frequency of a specific allele at a locus and spawning. This analysis considered Beaumaris or Menai Bridge as populations, and spawners and non-spawners as subpopulations within them. The sequential Bonferroni technique (Hochberg 1988) with an $\alpha = 0.05$ was used to establish whether a particular test was significant when considering multiple tests of the same hypothesis.

RESULTS

Between the 28th of January and the 12th of June 1994, a total of 658 mussels was subjected to spawning induction treatment and 235 spawned (Table 1). There were minor differences in the number of spawners from the Beaumaris (B) and Menai Bridge (MB) populations during the season. By the end of January the mussels were becoming sufficiently ripe to spawn following artificial induction. The number of spawners increased as the season progressed with a peak between mid March and the end of April. Spawning activity had ceased by the beginning of June.

The mean shell lengths of mussels used in these trials were between 57.2 and 66.2 for Beaumaris and between 56.6 and 65.0 mm for Menai Bridge (Table 1). Two-way ANOVAs for both sites showed no significant difference in mussel shell length between spawners and non-spawners for Menai Bridge ($F_{1,249} = 0.96$, ns),

TABLE 2.

***Mytilus edulis*. Sample size (n), agreement with the Hardy-Weinberg expectancies (HW), fixation index *Fis* and unbiased heterozygosity.**

		Jan 28		Feb 11		Feb 25		Mar 12		Mar 25		Apr 10		Apr 13		Apr 27		May 12		May 27	
Locus		**spa**	**nsp**	**spa**	**nsp**	**spa**	**nsp**	**spa**	**nsp**	**spa**	**nsp**	**spa**	**nsp**	**spa**	**nsp**	**spa**	**nsp**	**spa**	**nsp**	**spa**	**nsp**
	n	7	10	6	10	6	10	10	10	11	9	13	8	13	7	15	10	13	10	12	10
Est-D	HW	—	ns	ns	ns		—	ns	—	—	—	ns	—	ns		ns	ns	—	ns	ns	ns
	Fis		0.000	0.000	−0.125			−0.059				0.000		1.000		0.000	0.000		0.000	−0.100	0.000
Gpi	HW	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Fis	−0.263	−0.216	−0.290	−0.016	−0.250	−0.008	−0.286	0.196	0.265	0.360	0.072	−0.307	0.544	−0.111	−0.217	0.204	0.213	0.044	0.130	0.031
Lap	HW	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Fis	0.415	−0.167	−0.081	0.118	−0.389	−0.235	0.100	0.153	−0.301	−0.191	0.012	−0.018	−0.005	0.625	−0.345	0.153	0.238	0.082	0.429	0.126
Dia	HW	—	ns		ns	ns	ns		ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns
	Fis		0.000		−0.080	−0.250	0.640	1.000	0.561	−0.026	0.771*	0.000		0.294	0.000	0.243	−0.029	0.040	0.386	0.283	0.000
Gsr	HW	ns			ns	ns	ns	--	ns	ns	ns	—	ns	ns	ns	ns	ns	ns	ns		
	Fis	−0.043			−0.059	0.091	0.040		0.000	−0.096	−0.143		0.000	−0.021	0.000	−0.037	0.000	−0.091	0.000		
Ipp	HW	—	—	—	ns	—	ns	ns	ns	—	ns	ns	ns	-	--	ns	ns	—	—	—	ns
	Fis				0.000		−0.029	0.000	0.000		0.000	0.000	0.000			−0.037	0.000				0.000
Odh	HW	ns	ns		—		ns	ns	ns	ns	ns	ns	-	ns	ns	ns	ns	ns	ns	ns	
	Fis	−0.043	0.000				−0.059	0.654	1.000	0.643	−0.333	0.647		0.256	0.647	0.000	−0.080	−0.067	−0.080	0.185	
Pgm	HW	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Fis	0.400	0.109	0.615	−0.080	0.750	−0.059	0.012	0.557	0.279	−0.021	0.527*	−0.207	0.213	0.442	0.811	0.333	0.247	0.612	−0.048	0.217
Mpi	HW	—		-	ns		ns	-	ns	ns	ns	—	—	-	ns	ns		ns	ns	ns	ns
	Fis				−0.059		−0.059		−0.059	−0.053	0.143				−0.043	0.000		−0.043	−0.029	−0.023	0.000
Pgd	HW		—	--	ns	ns	ns	ns	ns	ns	—	ns	ns	—	ns	ns	ns	ns	ns	ns	
	Fis				−0.059	0.000	−0.059	0.000	−0.059	−0.053		0.000	−0.037		0.000	0.000	0.000	−0.021	−0.059	−0.023	
Overall	*Fis*	0.118	−0.069	0.038	−0.023	0.029	−0.014	0.238	0.329	0.088	0.093	0.233	−0.165	0.273	0.243	0.018	0.145	0.127	0.211	0.173	0.176
	− ve	3	2	2	7	3	7	2	2	5	4	0	4	2	2	4	2	4	3	4	0
	+ ve	2	1	1	1	2	2	4	5	3	3	4	0	5	3	2	3	4	4	4	4
H		0.214	0.200	0.167	0.310	0.283	0.360	0.216	0.210	0.255	0.344	0.185	0.250	0.200	0.200	0.233	0.220	0.285	0.250	0.217	0.190
s e		0.088	0.083	0.086	0.071	0.100	0.088	0.079	0.053	0.071	0.088	0.071	0.099	0.068	0.065	0.086	0.053	0.062	0.058	0.053	0.080
t		0.011		−0.126		−0.056		0.006		−0.089		−0.068		0.000		0.011		0.040		0.029	

(H) from mussels collected during the 1994 spawning season in Beaumaris, North Wales. spa = spawner, nsp = non-spawner, ns = not significant, * = significant following Bonferroni correction. —, test not carried out due to presence of fixed allele. s e standard error. *t* value of the difference between heterozygosities of spawners and non-spawners.

TABLE 3.

***Mytilus edulis*. Sample size (n), agreement with the Hardy-Weinberg expectancies (HW), fixation index *Fis* and unbiased heterozygosity (H) from mussels collected during the 1994 spawning season in Menai Bridge, North Wales.**

		Jan 28		Feb 11		Feb 25		Mar 12		Mar 25		Apr 27		May 12		May 27	
Locus		spa	nsp	spa	nsp	spa	nsp	spa	nsp	spa	nsp	spa	nsp	spa	nsp	spa	nsp
	n	3	10	6	10	11	9	10	10	12	9	14	10	13	7	10	10
EsD	HW	—	ns	—	—	—	—	—			—	ns	ns	ns	—	—	—
	Fis		−0.059						0.000	0.656		−0.020	0.000	0.000			
Gpi	HW	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Fis	1.000	0.118	0.318	−0.008	0.085	−0.116	0.204	0.273	0.106	−0.080	−0.111	0.153	−0.168	−0.220	−0.426	
Lap	HW	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Fis	−0.500	−0.273	−0.282	−0.256	−0.176	0.510	−0.426	−0.016	0.009	−0.091	0.025	0.455	0.034	−0.075	−0.116	0.023
Dia	HW	—	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	—	ns	ns
	Fis		−0.125	−0.111	−0.029	−0.081	−0.067	0.640	0.654	−0.158	−0.059	−0.213	−0.200	0.617		0.769	0.757
Gsr	HW	—	ns	—	ns	—	ns	ns	ns	ns	ns	ns	ns	ns	—	—	ns
	Fis		−0.029		−0.059		0.000	−0.059	0.000	0.000	0.000	0.000	−0.029	−0.043			0.080
Ipp	HW	ns	ns	—	ns	ns	—	ns	ns	ns	ns	—	ns	ns	—	—	ns
	Fis	0.000	0.000		0.000	0.000		0.000	0.000	0.304	0.000		0.000	0.000			0.000
Odh	HW	ns	ns	ns	ns	ns	ns	ns	—	ns	ns	ns	ns	ns	—	ns	ns
	Fis	1.000	−0.059	0.000	−0.059	−0.071	0.158	−0.029		0.200	−0.059	0.172	1.000	0.000		−0.108	0.000
Pgm	HW	ns	ns	—	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Fis	0.500	0.000		0.280	−0.176	0.294	0.446	0.100	−0.213	−0.001	−0.044	0.424	0.298	0.357	0.400	0.191
Mpi	HW	—	ns	—	ns	—	—	ns	ns	—	ns	ns	ns	ns	—	ns	—
	Fis		−0.080		0.000			−0.029	0.000		0.000	0.436	−0.077	−0.043		−0.059	
Pgd	HW	—	ns	ns	ns	—	—	ns	ns	—	—	ns	—	ns	—	ns	ns
	Fis		1.000	−0.250	0.000			0.000	0.000			0.000		0.000		−0.029	0.000
Overall	*Fis*	0.444	0.010	−0.037	0.012	−0.059	0.187	0.124	0.145	0.067	−0.048	0.019	0.253	0.117	0.059	0.098	0.053
	− ve	1	6	3	5	4	2	4	1	2	5	5	3	3	2	5	3
	+ ve	3	2	1	1	1	3	3	3	5	0	3	4	3	1	2	2
H		0.167	0.320	0.233	0.280	0.245	0.211	0.260	0.240	0.245	0.222	0.310	0.215	0.263	0.171	0.290	0.240
s.e.		0.102	0.076	0.094	0.076	0.082	0.073	0.076	0.083	0.072	0.064	0.078	0.051	0.083	0.095	0.097	0.082
t		−0.101		−0.038		0.034		0.018		0.025		0.093		0.099		0.039	

spa = spawner, nsp = non-spawner, ns = not significant, * = significant following Bonferroni correction, — test not carried out due to presence of a fixed allele, s.e. standard error, *t* value of the difference between heterozygosities of spawners and non-spawners.

but a significant difference for Beaumaris ($F_{1,307} = 10.091$, $p = 0.001$), with spawners being smaller. Overall (considering both sites), the mean shell length of the spawners was larger than that of the non-spawners in 6 instances out of 18. This ratio was not significantly different from a 1:1 ratio ($p > 0.1$) using the sign test (Sokal & Rohlf 1995). No significant difference in mussel length between sampling dates was found in Beaumaris ($F_{9,307} = 1.88$, ns), whilst there was a difference in Menai Bridge ($F_{7,249} = 3.39$, $p = 0.002$); however, this difference was probably a sampling effect because mussels were only roughly size-selected during collection. There was no tendency for mussels of a particular size to spawn for each sampling date and at each site, because there was no significant interaction in any of the two-factor ANOVAs (B: $F_{9,307} = 1.71$, ns; MB: $F_{7,249} = 1.76$, ns).

Agreement with HW expectations (by exact test) was found at most loci in most samples, except in one out of 152 cases for Beaumaris (*Dia*, March 12 spawners). This value represents only 0.65% of the total number of tests of the Hardy-Weinberg. Also whenever *Fis* was significantly different from zero at a particular locus it was always positive (5 cases in Beaumaris and 1 in Menai Bridge, Table 2 and Table 3) indicating too few heterozygotes. In one case (March 12 non-spawners, Table 2) there was a significant positive value of *Fis* across all loci indicating a cumulative heterozygote deficit in this group of 10 mussels.

The mean unbiased heterozygosity (*H*) across loci ranged from 0.167 to 0.360 in Beaumaris and from 0.167 to 0.32 in Menai Bridge. There were no significant differences between the *H* of spawners and non-spawners in any trial for either population (10 tests for Beaumaris and 8 for Menai Bridge, Table 2 and Table 3).

The sign test was used to determine whether there was a difference between the number of negative and positive values of the *Fis*. Firstly, +*Fis* and −*Fis* values for spawners and non-spawners were separately added for all loci at each sampling date (column wise - given at foot of Table 2 and Table 3). It was not possible to say that there were significant differences in any case. Secondly, the *Fis* values were added horizontally for each locus between spawners and non-spawners in both sites (Del Rio-Portilla 1996). Not a single significant difference was found after Bonferroni adjustment. Finally, total *Fis* values were assessed, and the negative percentages were 44.1% for spawners and 51.6% for non-spawners in Beaumaris, whilst in Menai Bridge, the *Fis* + ve values were 43.8% and 37.2% for spawners and non-spawners, respectively. None of these was significantly different from 50%. Furthermore, Wilcoxon tests showed that no median of the *Fis* values was different from a value of zero (B: spawners C (Wilcoxon statistic) = 639, ns; non-spawners C = 705, ns; MB: spawners C = 509, ns; non-spawners C = 452, ns)

Chi-square contingency table analyses were carried out on allele frequency data at each locus for each trial date and for both sites. In no case was there a significant difference in allele fre-

TABLE 4.

Mytilus edulis. Allele frequency, fixation index *Fis* and agreement with the Hardy Weinberg (HW) model from pooled data of mussel samples taken from Beaumaris.

Locus		spa	nsp	Locus		spa	nsp
EsD	n	106	94	*Ipp*	n	100	85
	74	0.000	0.000		85	0.020	0.035
	83	0.033	0.016		100	0.980	0.953
	100	0.953	0.963		119	0.000	0.012
	117	0.014	0.021				
	133	0.000	0.000				
	HW	ns	ns		HW	ns	ns
	Fis	0.174	−0.024		*Fis*	−0.016	−0.034
Gpi	n	106	94	*Lap*	n	106	94
	77	0.033	0.016		82	0.019	0.032
	83	0.038	0.043		90	0.127	0.096
	90	0.222	0.239		100	0.627	0.644
	93	0.047	0.090		108	0.208	0.207
	100	0.604	0.548		117	0.019	0.021
	104	0.057	0.064				
	112	0.000	0.000				
	HW	ns	ns		HW	ns	ns
	Fis	0.026	0.023		*Fis*	−0.014	0.026
Odh	n	106	94	*Pgm*	n	105	94
	58	0.005	0.000		66	0.005	0.000
	73	0.009	0.000		78	0.014	0.059
	82	0.071	0.074		86	0.052	0.096
	91	0.009	0.011		89	0.067	0.032
	100	0.877	0.894		100	0.676	0.622
	114	0.024	0.021		110	0.067	0.085
	130	0.005	0.000		115	0.100	0.085
	144	0.000	0.000		121	0.010	0.021
					130	0.010	0.000
	HW	ns	ns		HW		
	Fis	0.290	0.134		*Fis*	0.461*	0.210
Dia	n	106	94	*Gsr*	n	106	94
	92	0.137	0.128		57	0.028	0.016
	100	0.816	0.840		75	0.033	0.053
	115	0.047	0.032		100	0.920	0.915
					121	0.019	0.016
	HW	*			HW	ns	ns
	Fis	0.309*	0.465*		*Fis*	0.010	0.006
Pgd	n	100	85	*Mpi*	n	100	85
	64	0.020	0.059		70	0.025	0.065
	100	0.950	0.924		80	0.000	0.006
	131	0.005	0.006		100	0.960	0.912
	162	0.025	0.006		116	0.010	0.018
	213	0.000	0.006		150	0.005	0.000
	HW	ns	ns		HW	ns	ns
	Fis	−0.032	−0.060		*Fis*	−0.026	−0.002
Overall	*Fis*	0.168*	0.104*				

spa = spawner, nsp = non-spawner, n = number of mussels scored for that particular locus, adjustment to HW model: ns = non significant, * significant after Bonferroni correction.

TABLE 5.

Mytilus edulis. Allele frequency, fixation index *Fis* and agreement with the Hardy-Weinberg (HW) model from pooled data of mussel samples taken from Menai Bridge.

Locus		spa	nsp	Locus		spa	nsp
EsD	n	79	75	*Ipp*	n	80	75
	74	0.006	0.007		85	0.038	0.033
	83	0.019	0.020		100	0.956	0.960
	100	0.962	0.973		119	0.006	0.007
	117	0.006	0.000				
	133	0.006	0.000				
	HW	ns	ns		HW	ns	ns
	Fis	0.322	−0.015		*Fis*	0.115	−0.029
Gpi	n	80	75	*Lap*	n	79	75
	77	0.006	0.027		82	0.006	0.033
	83	0.050	0.027		90	0.095	0.147
	90	0.244	0.232		100	0.614	0.587
	93	0.069	0.044		108	0.234	0.207
	100	0.575	0.647		117	0.051	0.027
	104	0.044	0.033				
	112	0.013	0.000				
	HW	ns	ns		HW	ns	ns
	Fis	0.070	0.003		*Fis*	−0.108	0.049
Odh	n	80	75	*Pgm*	n	80	75
	58	0.006	0.000		66	0.006	0.000
	73	0.038	0.020		78	0.019	0.013
	82	0.063	0.040		86	0.063	0.073
	91	0.013	0.000		89	0.112	0.067
	100	0.850	0.887		100	0.587	0.553
	114	0.019	0.047		110	0.050	0.113
	130	0.006	0.000		115	0.131	0.173
	144	0.006	0.007		121	0.031	0.007
					130	0.000	0.000
	HW	ns	ns		HW	*	ns
	Fis	0.132	0.180		*Fis*	0.196	0.229*
Dia	n	78	75	*Gsr*	n	80	75
	92	0.160	0.093		57	0.006	0.007
	100	0.782	0.860		75	0.025	0.047
	115	0.058	0.047		100	0.962	0.920
					121	0.006	0.027
	HW	ns	ns		HW	ns	ns
	Fis	0.186	0.206		*Fis*	−0.023	−0.054
Pgd	n	80	74	*Mpi*	n	78	73
	64	0.019	0.007		70	0.045	0.034
	100	0.944	0.966		80	0.019	0.007
	131	0.006	0.014		100	0.936	0.945
	162	0.025	0.007		116	0.000	0.014
	213	0.006	0.007		150	0.000	0.000
	HW	ns	ns		HW	ns	ns
	Fis	−0.033	0.393*		*Fis*	0.164	−0.035
Overall	*Fis*	0.088	0.104*				

spa = spawner, nsp = non-spawner, n = number of mussels scored for that particular locus, adjustment to HW model, ns = non significant, * significant after Bonferroni correction.

quencies between samples of spawners and non-spawners suggesting no relationship between the possession of a particular allele and likelihood of spawning following artificial stimulus (Del Rio-Portilla 1996).

Data from all spawners were pooled, as were data from non-spawners, for each sample site and genetic analyses are given in Table 4 and Table 5. These pooled data showed agreements with the Hardy-Weinberg model at most of the loci, except in *Dia*, and *Pgm* in both spawners and non-spawners from Beaumaris, and *Pgm* of spawners in Menai Bridge. As with the unpooled data *Fis* was positive in all these cases of significant deviation from the model. Overall, the number of loci with positive values of *Fis* was 24 out of 40 tests which is not significantly different from a 1:1 ratio.

Heterozygosity was 0.23 (s.e. 0.061) and 0.26 (s.e. 0.063) for spawners and non-spawners in Beaumaris, and 0.26 (s.e. 0.070)

and 0.24 (s.e. 0.065) for spawners and non-spawners in Menai Bridge respectively. Neither of these differences between spawners and non-spawners was significant ($t = -0.353$, ns and $t = 0.038$, ns for Beaumaris and Menai Bridge respectively).

DISCUSSION

Artificial induction of spawning in the laboratory is currently the best test we can make of the ripeness of mussels in the wild. It could be argued that if one mussel (A) spawns in the laboratory following an artificial stimulus while another (B) does not, this does not prove that mussel A would spawn, and mussel B would not spawn, following a natural stimulus in the wild, however, there are no published reports of attempts to detect spawning mussels *in situ* and the practical problems associated with detecting, and collecting spawning individuals of bivalves in the wild for a study such as this are probably insurmountable. It is worth noting that in our trials the results of artificial mussel spawning induction during the spring agreed with previous observations of spawning in the wild in the Menai Strait (Bayne 1963; Seed 1976), with mussels having both an artificially induced and a natural spawning peak between April and May.

The data overall show a trend for mussels which spawned to be smaller than those which did not spawn and this effect is significant in the Beaumaris population. Possibly smaller mussels are more sensitive to the spawning stimulus but, more likely, this is a gender effect. Almost invariably more males than females spawn following the stimulus and this is particularly noticeable early in the season (Del Rio-Portilla 1996). Because of the reduced unit energetic cost of male vs. female gametes, males may spawn at a smaller size than females and this could account for the difference in size between spawners and non-spawners if we assume a 1:1 sex ratio in the sampled population.

Our results provide no evidence to support the hypothesis proposed by Zouros and Foltz (1984) that heterozygote deficiencies in natural populations of bivalves might be caused by genotype-dependent spawning. Allele frequencies at the scored loci were not different between spawning and non-spawning groups. Furthermore, average heterozygosities across all loci did not differ between groups and neither did an individual mussel's heterozygosity (the number of the scored loci at which it was heterozygous) relate to whether or not it spawned (Del Rio-Portilla 1996).

This is of interest because, according to Rodhouse et al. (1986), reproductive output (the number of gametes released) is related to individual heterozygosity in adult mussels larger than 49 mm and highly heterozygous mussels should therefore produce more offspring.

The overall samples from the Menai Bridge and Beaumaris populations do exhibit a similar pattern of heterozygote deficiencies to previous studies with other populations of mussels and with other species (Gosling & Wilkins 1985, Gaffney 1990). Although there were not significantly more instances of negative than positive values of *Fis*, and three of the four mean values of *Fis* were not significatively different from zero (Beaumaris: spawners mean 0.112 s.e. 0.036, $p < 0.01$; nonspawners mean 0.067 s.e. 0.028 n.s.; Menai Bridge: spawners mean 0.076 s.e. 0.040 n.s.; nonspawners mean 0.069 s.e. 0.034 n.s.), whenever significant deviations from the Hardy-Weinberg model did occur, they were always the result of too few heterozygotes. The loci showing heterozygote deficiencies in this study (based on exact test of H-W or significance of *Fis*, Tables 4 and 5 : *Dia, Pgd, Odh* and *Pgm*) have also shown heterozygote deficiencies in previous studies of mussels and other bivalves (Volckaert & Zouros 1989; Fairbrother & Beaumont 1993; Gaffney 1994; Hoare & Beaumont 1995; Ríos et al. 1996).

Although our results have demonstrated that the heterozygote deficiencies in these mussel populations have not arisen as a result of the timing of spawning being genotype-dependent, we have not proven that they are caused by any other factor. The presence of a differentiated sub-population structure (Wahlund effect), or consistent mating between close relatives (inbreeding) within our sampled populations seem very remote possibilities. More likely is the miscoring of null-allele heterozygotes as homozygotes. There is evidence that juvenile mussels which are heterozygous, or even homozygous for a null allele at the *Odh* locus are able to survive and are apparently at no disadvantage compared with other genotypes possessing active *Odh* alleles (Hoare & Beaumont 1995) and null alleles have been found at other loci in laboratory crosses of mussels and other bivalves (Foltz 1986a & Foltz 1986b; Gaffney 1994; Del Río & Beaumont, 2000). However, the *Odh* locus is probably a special case because this enzyme operates in a biochemical pathway which may not be essential to the organism (Hoare & Beaumont 1995). Although null alleles may have caused the observed significant heterozygote deficiencies at the *Odh* locus in this study, null alleles are thought less likely to be present at sufficiently high frequency to account for the significant deviations from the Hardy-Weinberg model at other loci such as *Pgm* and *Pgd* which operate within critical biochemical pathways (Zouros & Foltz 1984). All four sub-samples of mussels (spawners and non-spawners at 2 sites, Tables 4 and 5) showed high positive values of *Fis* for the *Odh* locus (mean *Fis* = 0.184) which would be expected if null alleles were present in the populations. This was also true for the *Pgm* and *Dia* loci (mean *Fis* = 0.274 and 0.292 respectively), but not for the *Pgd* locus which had negative values of *Fis* in three sub-samples.

Finally, selection against heterozygotes should also be considered as a potential cause of the heterozygote deficiencies. If selection is operating at a locus we would expect it to have a similar force and direction in our four sub-samples of mussels and this is true for *Odh, Pgm* and *Dia* loci which all have large positive *Fis* values, but not for the *Pgd* locus.

We conclude that the heterozygote deficiencies against the Hardy-Weinberg model detected in this study are probably the result of the presence of null alleles and/or selection against heterozygotes, but that they are not caused by genotype-dependent spawning time.

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ARTIFICIAL ENVIRONMENTAL CONDITIONS CAN AFFECT ALLOZYME GENETIC STRUCTURE OF THE MARINE GASTROPOD *PATELLA CAERULEA*

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ABSTRACT Samples of the Mediterranean limpet *Patella caerulea* collected from 10 sites were examined for genetic population structure. Six of the 20 identified enzymatic loci were polymorphic. The *AAT** locus was polymorphic only in two samples from an artificial environment (TI2 and PE). The proportion of polymorphic loci ranged from 0.20 to 0.30, and the observed and expected mean heterozygosity varied between 0.098 and 0.076 and between 0.109 and 0.086, respectively. Mean *Fis* values were significantly positive in *AAT**, ESTD *, *PEPC-2**, and *PEPD* , showing heterozygosity deficiency. In all, mean *Fst* value of 0.007 indicated high genetic homogeneity between the samples analyzed, whereas single-locus *Fst* analysis showed an interesting case of significant microscale genetic heterogeneity in *AAT** (*Fst* = 0.158, $P < 0.001$). The specimens collected from the two artificial environments (TI2 and PE) were responsible for *AAT** heterogeneity. These results, as suggested by other authors, could be related to the hydrodynamic conditions of artificial environments.

KEY WORDS: *Patella caerulea*, genetic variation, allozymes, artificial environment, Mediterranean Sea

INTRODUCTION

Patella caerulea L. is a very common gastropod along sheltered Mediterranean rocky shores, where it coexists with the congeneric species *P. rustica* L. and *P. ulyssiponensis* Gmelin. The Mediterranean sedentary *P. caerulea* presents a planktonic larval stage of uncertain length (Dodd 1957) and a high morphological variability (Bacci and Sella 1970, Sella et al. 1993) so that in some localities, shell coloration and morphology are very similar to those of *P. ulyssiponensis*.

The aim of the present paper is to analyze the genetic population structure of Mediterranean *P. caerulea* populations and also to compare natural and artificial samples to understand if allozyme polymorphism could be related to particular environmental conditions. Although some allozyme variation can be due to random genetic drift, good examples exist of natural selection acting on allozyme diversity. Allozyme heterozygosity and growth rate have been correlated in many molluses, even though the phenomenon is not universal (Volkaert & Zouros 1989) and natural selection acting on particular loci has been demonstrated in different organisms (Koehn et al. 1980, Hilbish et al. 1982, Altukhov 1990, Powers et al. 1991, Riddoch, 1993, Johannesson et al. 1995). Moreover, several authors (Karl & Avise 1992, Pogson & Zouros 1994) have compared different markers to analyze population genetic structure and have concluded that in some cases, allozyme diversity was dependent on natural selection.

MATERIALS AND METHODS

A total of 506 *P. caerulea* specimens was collected from the intertidal zone at eight sampling sites: Trieste (TR), Augusta (AU),

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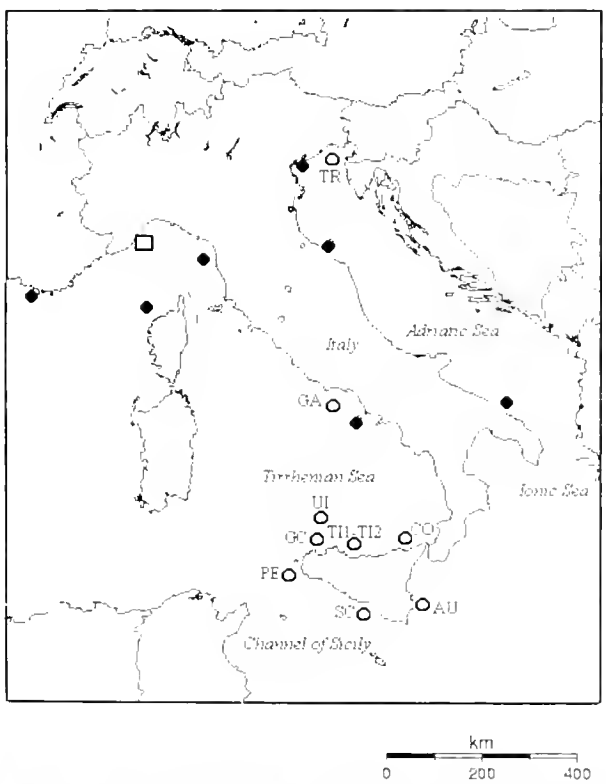

Figure 1. Sample sites. ■, present paper sample sites; TR, Trieste; AU, Augusta; SC, Scoglitti; CG, Capo Gallo; TI1, Termini Imerese 1; UI, Ustica Island; CO, Capo d'Orlando; GA, Gaeta; TI2, Termini Imerese 2; PE, Petrosino; ●, Badino et al. 1986 sample sites; □, Sella et al. 1993 sample sites.

TABLE 1.

nzyme commission number for the loci analyzed.

Enzymatic Systems	Loci
Sorbitol dehydrogenase (1.1.1.14)	*SDH-1,2**
Malate dehydrogenase (1.1.1.37)	*MDH-1,2,3**
Malic enzyme (1.1.1.40)	*ME**
Xanthine dehydrogenase (1.2.1.37)	*XDH**
Superoxide dismutase (1.15.1.1)	*SOD-1,2,3**
Aspartate aminotransferase (2.6.1.1)	*AAT**
Hexokinase (2.7.1.1)	*HK**
Esterase (3.1.1.1)	*EST-1,2,3**
EsteraseD (3.1.—)	*ESTD**
Peptidase C (lys-leu) (3.4—)	*PEPC-1,2**
PeptidaseD (phe-pro) (3.4.—)	*PEPD**
Phosphoglucoisomerase (5.3.1.9)	*PGI**

TABLE 2.

Allele frequencies in *P. caerulea* populations. Sample size (N), allelic frequencies, observed (H_o) and expected (H_e) heterozygosity. (*): $P < 0.05$; (**) $P < 0.001$. TR: Trieste; AU: Augusta; SC: Scoglitti; CG: Capo Gallo; TI1: Termini Imeresel; CO: Capo d'Orlando; UI: Ustica Island; GA: Gaeta; TI2: Termini Imerese2; PE: Petrosino.

	Natural Environments								Artificial Environments	
Locus	TR	AU	SC	CG	TI1	CO	UI	GA	TI2	PE
VAT-1 (N)	45	60	16	34	50	50	54	50	77	56
100	1	1	1	1	1	1	1	1	0.766	0.866
115	0	0	0	0	0	0	0	0	0.234	0.134
H_o	0	0	0	0	0	0	0	0	0.312	0.161
H_e	0	0	0	0	0	0	0	0	0.361	0.234
Fis									0.136	0.315**
EST-D (N)	45	59	16	31	50	45	54	50	80	57
80	0.033	0.008	0.031	0.048	0.018	0	0	0.020	0.006	0
85	0	0	0	0	0	0	0	0	0	0.018
87	0.011	0	0	0	0.018	0	0	0.020	0.013	0.018
90	0.022	0.025	0	0	0	0.044	0	0.050	0.019	0.009
92	0.022	0.068	0.063	0.081	0.087	0.089	0.083	0.050	0.075	0.035
98	0	0	0.031	0	0	0	0	0	0	0
100	0.867	0.847	0.781	0.823	0.824	0.833	0.889	0.770	0.837	0.842
104	0.033	0.051	0.094	0.048	0.053	0.033	0.028	0.08	0.044	0.079
110	0.011	0	0	0	0	0	0	0.010	0.006	0
H_o	0.267	0.237	0.312	0.322	0.248	0.244	0.222	0.320	0.312	0.263
H_e	0.276	0.276	0.387	0.317	0.230	0.299	0.204	0.398	0.292	0.285
Fis	−0.075	0.142	0.198	−0.017	−0.021	0.181	−0.090	0.199**	−0.070	0.078
PEPC-2 (N)	15	40	12	22	38	30	28	47	44	22
80	0	0	0	0.045	0.064	0	0.107	0.053	0.080	0.114
88	0.090	0.120	0.148	0.182	0.132	0.033	0.125	0.149	0.114	0
96	0.210	0.190	0.018	0.159	0.178	0.180	0.071	0.234	0.193	0.205
100	0.406	0.463	0.500	0.341	0.427	0.437	0.429	0.351	0.443	0.364
105	0.124	0.091	0.167	0.136	0.062	0.170	0.107	0.096	0.057	0.227
110	0.170	0.136	0.167	0.114	0.109	0.180	0.089	0.085	0.08	0.068
114	0	0	0	0.023	0.028	0	0.071	0.032	0.034	0
120	0	0	0	0	0	0	0	0	0	0.023
H_o	0.667	0.818	0.640	0.727	0.631	0.666	0.643	0.766	0.741	0.545
H_e	0.800	0.823	0.798	0.774	0.710	0.807	0.675	0.779	0.754	0.800
Fis	0.200	0.006	0.200	0.104	0.120	0.200	0.171	0.028	0.085	0.300**
PEPD (N)	42	21	15	29	50	29	53	50	70	68
80	0.048	0	0	0	0	0	0.019	0.020	0.021	0.022
90	0.440	0.476	0.333	0.397	0.450	0.448	0.340	0.440	0.471	0.463
100	0.512	0.476	0.633	0.569	0.507	0.431	0.604	0.490	0.471	0.500
110	0	0.048	0.033	0.034	0.043	0.121	0.038	0.050	0.036	0.015
H_o	0.357	0.380	0.400	0.517	0.550	0.456	0.452	0.540	0.475	0.45
H_e	0.548	0.400	0.500	0.526	0.609	0.432	0.523	0.569	0.557	0.55
Fis	0.351	0.322	0.211	0.019	0.120	0.096	0.136	0.052	0.181	0.182
PGI (N)	45	60	13	32	51	46	54	50	81	55
80	0	0.025	0	0.016	0.009	0	0	0.010	0.012	0.009
90	0.089	0.067	0.038	0.109	0.100	0.109	0.120	0.100	0.099	0.136
100	0.900	0.875	0.962	0.875	0.891	0.891	0.880	0.890	0.883	0.845
110	0.011	0.033	0	0	0	0	0	0	0.006	0.009
H	0.200	0.233	0.077	0.250	0.214	0.217	0.204	0.220	0.185	0.309
H_e	0.184	0.230	0.077	0.226	0.220	0.196	0.214	0.200	0.212	0.269
Fis	−0.088	−0.014	0	−0.110	−0.098	−0.111	0.047	−0.102	0.128	−0.151
SOD-1 (N)	45	60	16	34	51	46	50	50	82	55
90	0.044	0.008	0	0	0	0	0	0	0	0
100	0.956	0.992	1	1	1	1	1	1	0.994	1
115	0	0	0	0	0	0	0	0	0.006	0
H_o	0.089	0.017	0	0	0	0	0	0	0.012	0
H_e	0.086	0.017	0	0	0	0	0	0	0.012	0
Fis	−0.035	0							0	

Scoglitti (SC), Capo Gallo (CG), Termini Imerese (TI1), Capo d'Orlando (CO), Ustica Island (UI), and Gaeta (GA). Two additional samples were collected from artificial environments: (i) a channel carrying discharge waters from the thermoelectric plant at Termini Imerese (TI2); and (ii) a tank collecting sea water to be directed to an aquaculture plant in Petrosino (PE; Fig. 1). Both tanks, 60 m^2 in volume and 2 m in depth, were above ground and were exposed to direct sunlight. Living specimens were transported in sea water to the laboratory where they were stored at −80°C until they were used for electrophoresis. The whole animal body was homogenized in distilled water, centrifuged at 25,000 *g* for 30 min, and the supernatant was used for electrophoresis. Details of polyacrylamide gel slab electrophoresis (PAGE) are given in Davis (1964). Locus and allelic nomenclature were according to Shaklee et al. (1990; Table 1). Buffer and staining procedures were adapted from Richardson et al. (1986). Genetic variation was es-

timated by calculating the percentage of polymorphism (0.99 criterion), the mean number of alleles per locus, and the observed and expected heterozygosity using the program GENEPOP (v. 1.2, Raymond & Rousset 1995). This program was also used to test for departure from Hardy-Weinberg equilibrium by the Markov chain method (Guo & Thompson 1992). The FSTAT program (v. 1.2, Goudet 1995) was used to calculate Weir and Cockerham's (1984) unbiased estimate of Wright's (1978) *F* statistics by means of permutational procedures to test for significance. Population pairwise comparisons for *AAT** locus were tested for heterogeneity of genotype distribution using GENEPOP, which calculates an unbiased estimation of the *P* value of a log-likelihood (G) based on exact test by the Markov chain method (Goudet 1995).

RESULTS

Twelve enzymatic systems were analyzed and 20 loci were scored (Table 1). Among them, six were polymorphic under the 0.99 criterion: *AAT**, *EST-D**, *PEPC-2**, *PEPD** *PGI**, and *SOD-1** (Table 2). Sample size, allelic frequencies, and observed and expected heterozygosities for each polymorphic locus are given in Table 2. Four loci (*ESTD**, *PEPC-2**, *PEPD**, and *PGI**) were polymorphic in each sample analyzed. *SOD-1** was polymorphic in the TR, AU, and TI2 samples. Artificial environments (TI2 and PE) showed an additional polymorphic locus, *AAT**, which occurred within two alleles (**100* and **115*, Table 2). The mean number of alleles per locus ranged from 1.5 in CO to 2.0 in TI2, while the proportion of polymorphic loci ranged from 0.20 in SC to 0.30 in TI2. The mean observed heterozygosity ranged from 0.098 (TI2) to 0.076 (UI), and the expected heterozygosity ranged from 0.109 to 0.086. Three loci showed a significant genotype frequency departure from *H-W* expectations: *AAT** in PE, *ESTD** in GA, and *PEPC** in PE. The corresponding fixation index (*Fis*) was significantly positive, showing a heterozygote deficiency (Table 2). Moreover, single-locus *Fis* averaged over all the samples was significantly positive in *AAT**, *ESTD**, *PEPC**, and *PEPD** loci, and *Fit* was significantly positive in *AAT**, *PEPC**, and *PEPD** (Table 3).

To examine the spatial distribution of allelic frequencies, the mean value of *Fst* was calculated among the sampled sites. In all, a genetic homogeneity was indicated by the 0.007 value. On the contrary, single-locus *Fst* revealed a significant heterogeneity in *SOD** (*Fst* = 0.021, $P < 0.05$) and a highly significant heterogeneity in the *AAT** locus (*Fst* = 0.158, $P < 0.001$; Table 3). The high value of *Fst* in the *AAT** locus was dependent on artificial samples as shown in *Fst* comparison between all population pairs (Table 4). The genetic heterogeneity in *AAT** locus was high in all natural vs. artificial comparisons, whereas genotype distribution was homogeneous between the artificial environments (TI2 and PE).

TABLE 3.

Summary of *F* statistics.

Locus	F_{IS}	F_{ST}
*AAT**	0.192*	0.158*
*ESTD**	0.058*	−0.001
*PEPC-2**	0.111*	−0.004
*PEPD**	0.166*	−0.001
*PGI**	−0.034	−0.004
*SOD-1**	−0.023	0.021
Mean	0.106*	0.007

* $P < 0.05$.

DISCUSSION

The genetic parameters reported in this study have different values with respect to those observed by Sella et al. (1993) for the same species collected from the Ligurian coast (Fig. 1). We have found similar mean number of alleles per locus, but smaller values of polymorphism and mean heterozygosity. These differences could be attributed either to the different electrophoretic systems used (polyacrylamide vs. starch) or to the higher number and different kind of loci analysed by us.

With regards to heterozygosity, the observed deficiency at different loci and in different populations is consistent with the data reported by Zouros and Foltz (1984) for several mollusc species. Different mechanisms could explain heterozygosity deficiency such as inbreeding, Wahlund effect, selective mortality of certain genotypes, and null alleles (Volckaert & Zouros 1989, Manuris et al. 1998). Inbreeding is a populational event and thus, should it be responsible for the heterozygote deficiency, we would observe the effect across all polymorphic loci. However, as some loci showed a negative value of F_{IS} (i.e., heterozygosity excess) and as no homozygotes for null alleles were observed, we can reject the above explanations as responsible for causing heterozygosity deficiency. We cannot discard the Wahlund effect hypothesis, since heterozygosity deficiency could be the result of localized population mixing with allelic frequency differences in some loci. Finally, it cannot be excluded that locus-specific selection may cause heterozygosity deficiency for some loci and not for others.

The mean F_{ST} value was very low (0.007, $P > 0.05$) and was comparable to those obtained by Badino et al. (1986), who found F_{ST} values lower than 0.01 in Thyrrenian and Adriatic *P. caerulea* populations (Fig. 1). This suggests that there is a high level of gene flow among different geographic areas and that *P. caerulea* population size is very large. Despite the sedentary adult stage supposed to favor genetic differentiation, the larval stage increases gene flow and could be responsible for the observed large-scale genetic homogeneity. In contrast, single locus F_{ST} analysis showed significant heterogeneity in *SOD-1** (F_{ST} = 0.021, $P < 0.05$) and highly significant heterogeneity in *AAT** (F_{ST} = 0.158, $P < 0.001$). Whereas *SOD-1** heterogeneity was caused by two rare alleles (**90* and **115*) and could be related to geographical distance, the highly significant F_{ST} value found in *AAT** revealed an interesting case of microscale genetic heterogeneity.

The *AAT** locus occurred as a single allele (**100*) in natural environments, but showed two alleles (**100* and **115*) in the samples taken from the two artificial basins (PE and TI2; Table 2). The **100* allele was the same found by Sella et al. (1993), whereas Badino et al. (1986) did not analyze the *AAT** locus. It is relevant that *AAT** allelic frequencies were very similar between TI2 and PE samples (sites about 150 km apart), whereas genetic heterogeneity was maintained even if the distance between samples did not exceed 1 km (TI1-natural vs. TI2-artificial, Table 4). Hence, despite the large-scale genetic homogeneity observed for the majority of polymorphic loci analyzed, the *AAT** locus showed a very highly localized heterogeneity. Significant heterogeneity among populations on a local scale, but little additional large-scale geographical variation, have been observed in several marine gastropods molluscs (Johnson & Black 1984, Johnson et al. 1993, Hurst & Skibinski 1995), and different mechanisms such as mixing of larvae or post-settlement selection have been invoked. As we found a high level of homogeneity at the majority of polymorphic

TABLE 4.

Pairwise *Fst* in *AAT* locus.

	TR	AU	SC	CG	TH	CO	UI	GA	TI2
TI2	0.187**	0.207**	0.138*	0.147*	0.180*	0.194**	0.199**	0.194*	–
PE	0.111**	0.128**	0.066	0.070	0.120*	0.117**	0.121**	0.117**	0.023

$^{**}P < 0.001$; $^{*}P < 0.05$.

loci, the reproductive isolation between coast and artificial environments as an explanation for the spatial differentiation in the *AAT** locus can be rejected. The inconsistency among the loci analyzed indicates that the cause for local heterogeneity is not dependent on breeding systems, but is locus specific.

As several authors suggest, allozymes do not always behave as neutral markers (Hilbish et al. 1982, Altukov 1990, Karl & Avise 1992, Riddoch 1993, Johannesson et al. 1995, Mitton 1997) and even though we were not able to demonstrate the functional relationship between allozyme variations and environmental parameters of artificial basins, we think that the strong genetic heterogeneity found in the *AAT** locus may be related to the peculiar environmental conditions. The first artificial sample site was the channel collecting the discharge water used to cool down the Termini Imerese thermoelectric plant (TI2). Salinity of the discharged water equals that of sea water (37‰), whereas temperatures are slightly higher (1°C to 2°C) than those measured in surrounding coastal waters. Hydrodynamic patterns are remarkably different as the site is sheltered from wave motion, though it is subjected to a continuous flow of discharge water. The second artificial site, located in an aquaculture plant in Petrosino (PE), is a tank collecting sea water to be directed to the breeding tanks. As result of the continuous exchange with the seawater, temperature and salinity do not differ much when compared to the natural sites, whereas the hydrodynamic patterns do, owing to the continuous flow of incoming water. Even if several authors invoked temperature (Powers et al. 1991, Sokolova & Portner 2001) and salinity (Koehn et al. 1980) as responsible for the particular genetic adaptation found in marine organisms, we cannot demonstrate this tendency because in our case study, temperature and salinity of artificial sample sites are the same of those of the sea. Microscale allozyme variation in the *AAT** locus was also found in the marine snail *Littorina saxatilis* (Johannesson et al. 1995), and the authors concluded that a strong natural selection was acting. Therefore, it is probable that the *AAT** locus was the specific target of complex environmental-genetic interactions. The *115** allele was probably maintained at low frequencies (below the limit of detection in a sample of 50–100 individuals) in natural environments by a mutation/selection balance, and it increases in frequency as a result of selection in artificial environments. Unfortunately, we do not know what kind of physiological consequences a locus could have. The *AAT** enzyme is involved in the degradation process of nitrogen-containing compounds and it is known that marine molluscs of the intertidal zone have developed different mechanisms of excretion of these compounds in relation to vertical zoning and to wave action exposure (Cognetti & Sarà 1981). The microscale variation in the *AAT** locus found in *P. caerulea* and in *L. saxatilis* could be related to some of these adaptive processes.

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EVALUATION OF MICROSATELLITE PRIMER CONSERVATION IN ABALONE

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ABSTRACT This study investigated the interspecific amplification of 22 microsatellite loci developed for *Haliotis rubra* across 12 other species within the Haliotidae. We reveal, through the discussion of three specific cases, a need for more thorough assays of microsatellite locus conservation, to ensure the utility of existing markers in foreign species. Optimization and analysis of PCR products revealed that of 12 loci examined in the Australian *H. laevigata*, only five were able to be reliably scored, while 6 of 10 for the South African *H. midae* were scoreable and none of the three for the North American species were useable. The assay examines five species from Australia, three from New Zealand, two from South Africa and two from North America. Amplification success varied from 68% for *H. conicopora*, a possible sub-species, to 14% for the distantly separated and related species from North America. The importance of cross-species amplification is discussed, but it is apparent that the proportion of markers that may be useful in other species of the same genera will vary greatly with the taxa being investigated.

KEY WORDS: *Haliotis*, abalone, microsatellites, aquaculture, primer conservation

INTRODUCTION

Members of the genus *Haliotis*, commonly called abalone, are distributed in coastal waters of all continents. Many of the 57 recognized species (Geiger 2000) are harvested commercially or recreationally, and they are a highly valuable marine resource. Abalone populations, like those of most highly prized marine resources, have come under increased legal and illegal harvesting pressures in recent years as demand for the product continues to rise, and methods for their capture and distribution are refined. Along with the expanding effort within abalone fisheries worldwide, and in some cases the decline of those fisheries, there has been extensive development in the culture of many abalone species (Oakes & Ponte 1996, McBride 1998, Cook 1998). An important technological advancement that will benefit both the culture and wild harvest industries is the development and application of molecular genetic markers.

Molecular genetic markers are widely used in many seafood industries for both wild and aquaculture needs; for example, in Salmonids (Reilly et al. 1999) and oysters (McGoldrick & Hedgecock 1996). They can be used for applications as diverse as tracking the biological history of populations (Chambers & MacAvoy 2000), or as specific as determining the parentage of individuals in culture (O'Reilly et al. 1998). One such marker that has been applied in other genera (Dallimer 1999, Wu et al. 1999, Nesje et al. 2000) but has only recently become popular in abalone research is microsatellite DNA. This marker consists of a nucleotide sequence of between two and six base pairs repeated in series at a set point on a chromosome (locus). The number of times that sequence is repeated at a single locus varies within (heterozygous individuals) and between individuals (intra-specific variation), and where that same locus is conserved across species, the number of repeats may vary widely (inter-specific variation) (Wright & Bentzen 1994).

Microsatellite markers have been developed from partial genomic libraries of four *Haliotis* species: *H. asinina* (Selvamani et al. 2000), *H. kamtschatkana* (Whithler, pers. comm.), *H. rubra* (Huang & Hanna 1998, Evans et al. 2000) and *H. rufescens* (Kirby et al. 1998). The development of microsatellite DNA markers, as described in each of these papers, is a time consuming and expensive process (Wright & Bentzen 1994). For these reasons, the efficacy of markers between species within the same genus or family have been examined in both plant and animal groups with ambiguous results (Dowling et al. 1996, Huang & Hanna 1998).

White and Powell (1997) tested 11 microsatellite markers developed for the hardwood, *Swietenia humilus* for conservation within 11 members of the Meliaceae family, representing seven genera. They detailed four species specific, one genus specific and three family wide markers. This trend of good marker conservation within plant families is supported by other studies such as that by Thomas and Scott (1993) who found that primer sequence conservation existed among grapevine species, and more recently by Rosetto et al. (2000) who showed similar sequence conservation among members of the Myrtaceae family.

Wirth et al. (1999) examined conservation of 11 microsatellite loci developed for the Walleye, *Stizostedion vitreum* in four species representing two genera of the Percidae family. Three of the markers were conserved in all species tested, two were found to be specific to *Stizostedion* genus, four produced amplification from both genera, but not all species within them, and the remaining two markers amplified only in *S. canadese*. Primer sequences have also shown some conservation across 10 species of four genera of Lemur, endemic to Madagascar (Jekielek & Strobek 1999).

Huang and Hanna (1998) considered the cross-species amplification of their three *H. rubra* microsatellite loci in species from USA (2 species), South Africa (2 species), South Korea (6 species) and Australia (5 species). Of the ten species tested from outside of Australia, only two of the South Korean species produced any amplification product. Within Australian species the markers were more conserved, with at least two of the three loci producing an amplification product in all Australian species tested, except for *Haliotis laevigata*, the greenlip abalone, which failed to amplify a product at any of the three loci. As *H. rubra* and *H. laevigata* are known to produce hybrids in the wild (Brown 1995) this latter result is unexpected, and required further examination as the hybrid is being developed as an aquaculture product for which molecular markers are required.

In this article we describe the cross-species amplification of 21 microsatellite loci (22 primer pairs) developed for use in the Aus-

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tralian Blacklip abalone, *Haliotis rubra* (Leach, 1814). Twelve species from Australia (5 species), New Zealand (3 species), South Africa (2 species) and North America (2 species) were tested. These 12 species come from two diserete phylogenetic clusters within the genus based on sperm lysin DNA sequences (Lee & Vacquier 1995). We then expand upon this work by describing the optimization of some of these loci for genetic variation research in wild and cultured South African *Haliotis midae*, pedigree analysis and genetic variation research with the Australian *H. laevigata*, and genetic variation studies in the North American abalone, *H. fulgens*.

MATERIALS AND METHODS

Microsatellite Amplification

The twelve abalone species tested in this study were chosen to provide both close and distant evolutionary relationships to *Haliotis rubra*. Four species (*H. conicopora* Péron, 1816; *H. laevigata* Donovan, 1808; *H. roei* Gray, 1826; *H. scalaris* Leach, 1814) share a temperate Australian habitat with *H. rubra*, while *H. asinina* (Linnaeus, 1758) is a tropical species form Australian waters. *Haliotis australis* (Gmelin, 1791), *H. iris* (Gmelin, 1791) and *H. virginea* (Gmelin, 1791) are temperate species from neighboring New Zealand. The two most prevalent South African species *H. midae* (linnaeus, 1758) and *H. spadicea* (Donovan, 1808) and two species of commercial importance in North America *H. corrugata* (Wood, 1828) and *H. fulgens* (Philipi, 1845) are examples of distant temperate species.

Twenty-two microsatellite primer pairs were developed for *Haliotis rubra* (Table 1) using methods described in Evans et al. (2000). Their potential for cross-species amplification was tested under standard PCR conditions with DNA extracted from gill or muscle tissue from at least two individuals of the 12 test species using a modified CTAB protocol (Grewe et al. 1993). Such low sample sizes are a problem for determination of diversity indices or population structure, but are sufficient for the detection of the locus in another species.

TABLE 1.

Characterization of 22 microsatellite primer pairs tested for cross-species amplification in this study. Previously published primers appear last, and a citation is given instead of full sequence.

Locus	Repeat Sequence	Primer Sequence (5′-3′) (F-Forward, R-Reverse)	Accession Number	Approx. Size in H. rubra (bp)
*cmrHr*1.5	$(CAGA)_5$	F-GGAAGAGGTATCGTAAACTG R-AGTCTCCCTGGTAAAACG	AF 302824	126
*cmrHr*1.6	$(CA)_4$ $(CA)_3$	F-GTTGTAAATGATGCCCTC R-CGTCTTTTTATTCAACGCC	AF 302825	89
*cmrHr*1.23	$(AC)_{32}$	F-GCTGGGAAATCAATCTTC R-CCTCACTTTCAACACTCAC	AF 302826	122
*cmrHr*2.3	$(GT)_{14}TT(TG)_3$	F-CCAGGCCCTATTCTTTCACA R-CGTCGCACTAAACACTGCAT	AF 302827	100
*cmrHr*2.5	$(GT)_{21}$	F-GCGCAGACATTCATCGGATA R-GTCCATCGTCGACAGGTTTT	AF 194955	283–299
**cmrHr*2.15	$(CA)_{27}$	F-TTTACATCGCATCGGCATTA R-TACTTAACGTTGCCCTGCCT	AF 195956	288
*cmrHr*2.17	$(GT)_{38}$	F-AGGACTTGCCCAACCTTTTT R-TTACAGAACAAACACAAGTATTGAA	AF 302828	226
*cmrHr*2.18	$(GAGT)_3$	F-GCTCCAGAATTCAAGGGTTG R-GCTGCTTAACCTCAGGATGC	AF 302829	134
*cmrHr*2.20	$(AC)_{23}(GCAC)_{18}$	F-TTTTGAATGATTGTATTTCGTTTT R-TACCTTGCATCGTAATAAACAGACAC	AF 302830	186
*cmrHr*2.22	$(CA)_{22}$	F-GGGTCGTCAGGTAGGTAGCA R-CCATAATCAGAGGGGAAGCA	AF 302831	117–193
*cmrHr*2.23	$(AC)_{16}$	F-TGGAAGCTTTTCAAACATTGG R-TACAATGGGGATTAAGAAGC	AF 302832	258–266
*cmrHr*2.27	$(GT)_{17}(GCGT)_{23}(GT)_2$	F-GTCCAGGTCCACAGCTCATT R-GGAATTGAAGACCCTCCTCC	AF 302833	347
*cmrHr*2.29	$(CA)_{58}$	F-TGATTGGTGTGTGAGGTGAAA R-CCGATGCCCTTATCATCACT	AF 302834	321
*cmrHr*1.11	$(AC)_{15}$	Evans et al., 2000	AF 194951	172–176
*cmrHr*1.14	$(GT)_{13}TT(GT)_2GA(GT)_3$	Evans et al., 2000	AF 195952	252–262
*cmrHr*1.24	$(AT)_8$	Evans et al., 2000	AF 195953	216–236
*cmrHr*1.25	$(CA)_{25}(AT)_6TT(AT)_5(TG)_3$	Evans et al., 2000	AF 195954	291–309
**cmrHr*2.9	$(GT)_{27}$	Evans et al., 2000	AF 195956	156–202
*cmrHr*2.14	$(GAGT)_8$ $(GAGT)_5$	Evans et al., 2000	AF 195957	209–235
*cmrHr*2.26a	$(ATTC)_5T_4C(ATTC)_2$	Evans et al., 2000	AF 195958	190–212
*cmrHr*2.30	$(GT)_6$ $(GT)_{13}(TG)_{12}(AG)_5$ $(TG)_3$ $(TG)_{16}$	Evans et al., 2000	AF 195959	284–328
*cmrHr*2.36	$(AC)_{21}$	Evans et al., 2000	AF 195960	83–121

The two primer pairs preceeded by the "*" both amplify the same locus, with *cmrHr*2.9 being internal to *cmrHr*2.15.

PCR reactions were performed in a volume of 25 µl consisting of 67 mM TrisHCl, pH 8.8; 16.6 mM $(NH_4)_2SO_4$; 0.45% Triton X-100; 0.2 mg/mL gelatin; 2.5 mM $MgCl_2$; 10 pmoles of each primer; 200 µM dNTPs; 0.5 U *Taq* F1 polymerase (Fisher Biotech); and ~ 20 ng genomic DNA template. Amplification was in a Perkin Elmer 9600 thermocycler with one cycle of 94 C for 1 min, 50 C for 15 s and 72 C for 1 min, followed by 30 cycles of 94 C for 15 s, 50 C for 15 s and 72 C for 1 min. These cycles were followed by a final extension step of 72 C for 10 min. Amplification products were visualized on 2% TBE agarose gels, and markers were scored as present when a single band of between 75 bp and 450 bp was detected (Table 2). Any amplification products above this size range, although possibly containing the microsatellite repeat unit, can not be scored on the ABI377 using the size standards commonly available. It is possible to score larger alleles with different systems, but such large products increase the possibility of mutation in the regions flanking the microsatellite rather than within the repeat unit. In such cases it would be best to design new primers closer to the repeat unit. Amplification products less than 75 base pairs in size are difficult to reliably score due to their proximity to such PCR artifacts as primer-dimer, and the presence of unincorporated dyes.

H. midae *Optimization*

The loci that produced an amplification product for *H. midae* under standard conditions were further examined to optimize PCR parameters. Samples were initially subjected to PCR amplification at temperatures of 50 C and 55 C and with DNA template concentrations of 10ng/µl and 2ng/µl. Amplification products for all ten markers were not improved by increasing the annealing temperature from 50 C to 55 C, but were improved by increasing the DNA template concentration to 10ng/µl in all samples. The markers were amplified in eight individuals of *H. midae* from Cape Hangklip on the southwest coast of South Africa. Products were diluted relative to amplification strength and mixed with formamide, loading dye and Genescan Tamra500 size standard (PE-Applied Biosystems), denatured at 95°C for 2 min and 1.2µl loaded onto a 4% denaturing polyacrylamide gel. Samples were run on an ABI377 DNA autosequencer and genotypes determined using Genpotyper® software. Allele variation was scored between the eight individuals.

H. laevigata *and* H. fulgens *Optimization*

All loci that produced an amplification product in one of these two species under standard amplification conditions were subjected to further testing for optimization. This included a range of annealing temperatures from 48°C to 58°C, "Touchdown-PCR" to improve primer specificity, where the annealing temperature at the beginning of the cycling program was high and was lowered by either 0.5 or 1.0°C each cycle until the lowest selected annealing temperature was reached. In addition, DNA template concentrations tested ranged from 1ng/µl to 30ng/µl, and $MgCl_2$ concentrations tested ranged from 1mM to 5mM. All loci were tested on at least 30 *H. laevigata* or 8 *H. fulgens* individuals.

RESULTS

Microsatellite Amplification

Nineteen of the twenty-two primer pairs tested successfully amplified a product in at least one species other than *H. rubra* (Table 2). Not surprisingly, the species that appears to have retained the most loci, at 15, is *H. conicopora*, a species that has been touted as perhaps a sub-species of *H. rubra* (Geiger, 2000). The three other temperate Australian species (*H. laevigata*, *H. scalaris*, *H. roei*) produced an amplification product from twelve of the 22 primer pairs. *Haliotis asinina*, the only tropical species included in the study showed sequence conservation in only five of the markers tested. The three species from New Zealand showed conservation of 5, 5, and 9 markers for *H. iris*, *H. australis* and *H. virginea* respectively. As expected there was little cross-species amplification seen in the North American species *H. rufescens* and *H. fulgens* (three markers each), which were shown to be in a distant clade to *H. rubra* by Lee and Vacquier (1995). It should be noted that the three loci producing an amplification product in the two North American species were the only loci to amplify a product in all 12 species tested. Interestingly though, the South African species showed more sequence conservation than the species from New Zealand, with ten markers being conserved in *H. midae* and nine in *H. spadicea*. None of the primer pairs was shown to be specific to all Australian species or to particular climatic regimes such as temperate and tropical species. Some primer pairs produced an amplification product that was dramatically different in size to that expected. Where that product was greater than 450 or less than 75 base pairs the marker was denoted by an "a" for altered product size. Although these altered products may contain the same microsatellite as other amplification products, they can be of no use if they can't be scored reliably.

Previously, researchers have simply reported agarose gel detection as the retention of a locus in a related species. In this article, we attempt to bridge the gap between identifying the presence of a marker in a related species, and the use of that marker for further research. We present here, three case studies, in which we have taken the markers identified from the preliminary screening tests and attempted to optimize them for routine research in *Haliotis midae*, *H. laevigata*, and *H. fulgens*.

Haliotis midae—*the South African Abalone*

The 10 loci shown to produce an amplification product from *H. midae* in the preliminary screening were: *cmrHr*1.23, *cmrHr*1.24, *cmrHr*2.9, *cmrHr*2.15, *cmrHr*2.20, *cmrHr*2.23, *cmrHr*2.27, *cmrHr*2.29, *cmrHr*2.30, *cmrHr*2.36 (Table 2). At the initial PCR amplification conditions, both *cmrHr*2.27 and *cmrHr*2.30 produced non-specific products when examined using the more sensitive automated detection techniques. These loci were re-amplified at annealing temperatures of between 50C and 58C and with $[MgCl_2]$ of between 2.0 and 3.0 mM. The resultant amplification products however were also non-specific and these markers were not examined further.

Two of the markers were monomorphic in the eight individuals examined and were not tested further in this study. These markers were *cmrHr*1.24 and *cmrHr*2.27. It is unlikely that these markers are actually the same locus as that amplified in *H. rubra*, as they were somewhat smaller than the product produced in that species, and did not show characteristic microsatellite amplification profiles. The variation at each of the remaining 6 loci ranged from the minimum of 2 alleles at *cmrHr*2.30 (226–242 bp), 3 alleles at *cmrHr*2.23 (244–252 bp), 4 alleles at *cmrHr*2.36 (101–119 bp), 5 alleles at *cmrHr*2.15 (250–280 bp), 6 alleles at *cmrHr*2.9 (173–205 bp) and a maximum of 8 alleles at *cmrHr*2.29 (425–469 bp).

TABLE 2.

Cross-species amplification using primers designed for *Haliotis rubra*.

	cmrHr	1.5	1.6	1.11	1.14	1.23	1.24	1.25	2.3	2.5	2.9	2.14	2.15	2.17	2.18	2.20	2.22	2.23	2.26	2.27	2.29	2.30	2.36	Total
Australia	*H. rubra*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	22
	H. laevigata	–	+	–	+	–	+	a	+	a	–	+	–	+	a	+	+	+	–	+	+	+	–	12
	H. scalaris	–	+	–	–	+	+	–	+	+	–	+	–	+	a	+	–	+	–	+	+	+	–	12
	H. conicopora	+	+	+	+	–	+	–	+	+	+	+	+	+	–	+	a	+	–	+	+	+	–	15
	H. roei	+	–	–	+	–	+	–	+	a	+	+	+	–	–	+	a	+	a	+	+	+	–	12
	H. asinina	–	–	–	–	+	a	–	+	a	–	–	–	–	–	+	a	–	–	+	–	+	–	5
New Zealand	*H. iris*	–	–	–	–	–	a	–	+	–	–	–	–	–	–	+	a	a	–	+	–	+	+	5
	H. australis	–	–	+	–	–	a	–	+	–	–	–	–	–	–	+	–	a	–	+	–	+	–	5
	H. virginea	–	–	–	–	–	a	–	+	+	+	+	+	–	–	+	–	+	–	+	–	+	–	9
South Africa	*H. midae*	–	a	–	–	+	+	–	a	a	+	–	+	–	–	+	–	+	–	+	+	+	+	10
	H. spadicea	+	+	–	–	+	a	–	+	+	–	–	+	–	–	+	–	–	–	+	–	–	+	9
North America	*H. fulgens*	–	–	–	–	–	–	–	+	a	a	–	–	–	–	+	a	–	–	+	–	–	–	3
	H. corrugata	–	–	–	–	–	–	–	+	a	a	–	–	–	–	+	–	–	–	+	–	–	–	3
	Total	4	5	3	4	5	6	1	12	5	5	6	6	3	1	13	2	7	1	13	6	10	4	

Assays producing a PCR product of expected size are indicated by +, those producing multiple bands or no product as –, and those producing bands of an altered size to that expected are represented by an "a".

Haliotis laevigata—*the Greenlip Abalone*

Of the 12 markers identified as being conserved in *H. laevigata* (Table 2), only five proved to be reliable for further studies after evaluation in 20 greenlip individuals. The reasons for exclusion of the other seven markers are listed in Table 3, but were due to either non-specific or unreliable amplification. Touchdown PCR cycles failed to clean up the peak profiles of locus *cmrHr*2.22. Two loci, *cmrHr*1.6 and *cmrHr*1.24 were monomorphic at 81 and 228 bp respectively in the 12 individuals examined, while the remaining three loci were variable with 7, 6 and 7 alleles detected for *cmrHr*2.14, *cmrHr*2.23 and *cmrHr*2.30 respectively.

Haliotis fulgens—*the Blue Abalone from Mexico*

The preliminary screening process identified three markers that produced an amplification product. These markers were *cmrHr*2.3, *cmrHr*2.20 and *cmrHr*2.27 (Table 2). PCR amplification products were however, always non-specific when visualized on agarose gels after all optimization conditions. Each product consisted of multiple bands within a small size range, such that allele identification was unreliable.

DISCUSSION

The development of microsatellite markers is known to be both expensive and time consuming (Wright & Bentzen, 1994). Many researchers that have produced microsatellite markers for their species have therefore examined the applicability of those markers to similar questions in related species. The use of agarose gel detection of PCR products has been utilized for the estimation of microsatellite loci conservation across species by researchers of other taxa (White & Powell 1997, Isagi et al. 1999). Huang and Hanna (1998), Wirth et al. (1999) and Rossetto et al. (2000), however examined these products further by denaturing polyacrylamide gel electrophoresis (PAGE) methods which reveal allele sizes and genotypes. Others have sequenced the markers in the new species to ensure that the locus being amplified does indeed match that expected (Ezenwa et al. 1998).

Whilst the screening of microsatellite markers in related species by sequencing techniques is obviously the most thorough method to determine marker conservation, it is also very expensive and time consuming. In instances where large numbers of microsatellite markers are being screened across many related species for which markers are not immediately required, this process may be considered to be excessive. Likewise, the optimization of primer pairs for genotyping through either radioactive labels or fluorescence primed, automated detection techniques is also time consuming and expensive. For this reason we took the simplest approach to determining marker conservation within abalone and have then followed this by detailing efforts to optimize the markers identified in the preliminary screening for use in other species.

Our research shows that a simplistic approach such as our initial screening can lead to a misleadingly high number of markers appearing to be conserved in related species. We report a 60% (6 from 10) success rate in the optimization of *H. rubra* markers for *H. midae*, 25% (3 from 12) for *H. laevigata* and 0% (0 from 3) in *H. fulgens*. Whilst the testing of molecular markers in related species is an important component in the sharing of information, it should be noted that simply determining that a product of similar size can be amplified in another species does not suggest that marker will be useful for that species. The three markers suitable for further studies of *H. laevigata* were *cmrHr*2.14, *cmrHr*2.23 and

TABLE 3.

Microsatellite markers initially identified as conserved in *H. laevigata*, and reasons for their exclusion from further research.

Locus	Accession numbers	Repeat Sequence	Reasons for exclusion
*cmrHr*1.6	AF 302828	$(CA)_4$ $(CA)_3$	**Monomorhic in H. laevigata**
*cmrHr*1.14	AF 194952	$(GT)_{13}TT(GT)_2GA(GT)_4$	Non-specific
*cmrHr*1.24	AF 194953	$(AT)_8$	**Monomorhic in H. laevigata**
*cmrHr*2.3	AF 302827	$(GT)_{14}TT(TG)_3$	Non-specific
*cmrHr*2.14	AF 195957	$(GAGT)_8$ $(GAGT)_5$	**Suitable for research**
*cmrHr*2.17	AF 302828	$(GT)_{38}$	Non-specific
*cmrHr*2.20	AF 302830	$(AC)_{23}(GCAC)_{18}$	Unreliable amplification
*cmrHr*2.22	AF 302831	$(CA)_{22}$	Unscoreable-very messy peaks
*cmrHr*2.23	AF 302832	$(AC)_{16}$	**Suitable for research**
*cmrHr*2.27	AF 302833	$(GT)_{17}(GCGT)_{23}(GT)_2$	Unreliable amplification
*cmrHr*2.29	AF 302834	$(CA)_{58}$	Unreliable amplification
*cmrHr*2.30a	AF 195959	$(GT)_6$ $(GT)_{13}(TG)_{12}(AG)_5(T)$	**Suitable for research**

Sample size for testing was n = 12.

*cmrHr*2.30. Two of these three markers have been used to determine broodstock contributors in controlled spawning of *H. laevigata*/*H. rubra* hybrids at a commercial culture facility (unpublished data). Locus *cmrHr*2.23 was not useful in the hybrid study however, as although it was easy to score in *H. laevigata*, a third allele of equal intensity to the first two was detected in some *H. rubra* samples, and in many of the hybrid progeny.

It should also be noted that the optimal conditions for PCR amplification would vary dramatically with different thermal cyclers. This was exemplified in the transfer of 6 *H. rubra* microsatellite loci that produced clean PCR products in *H. midae* in our study, but required extensive re-optimization when used in a genetic variation study in Cape Town, South Africa (Evans et al. in prep.). The most obvious reason for this discrepancy was the large variation in ramp times between the respective PCR machines. For this reason any attempt to transfer molecular marker technology between laboratories, and particularly between species will require additional PCR optimization at the new site. The substitution of specified reagents with those that are cheaper or more readily available may also affect amplification (Evans et al. in prep.).

One thing that is often overlooked when testing microsatellite primers is the design of the primer sites. The failure of a particular locus to amplify in another species may not mean that the microsatellite repeat is not present in that species, but simply that one or both of the primer sites have not been conserved. As the majority of microsatellite primers are published as part of a larger sequence on the Genbank (NCBI) database, the option of primer re-design is available. In this study, we have designed two pairs of primers for the *cmrHr*2.9 clone, with the *cmrHr*2.9 primers being internal to those of *cmrHr*2.15. What we have seen in this case is that the external primers (*cmrHr*2.15) were conserved in the South African species, *H. spadiceae*, while the internal primers (*cmrHr*2.9) were not (Table 2). It could be argued therefore that the examination of published sequences, and if necessary the re-design of primer sites, could be a more affordable solution to marker development than the creation of a new microsatellite library.

This study shows that microsatellite loci isolated from Australian blacklip abalone, *H. rubra*, can be amplified in some related *Haliotis* species, but that the likelihood of marker conservation is reduced with increasing phylogenetic distance. Rosetto et al. (1999) suggest that the selection of a single species from a large genera for microsatellite locus development will result in a suite of markers for most taxa in that genus. They detail only minimal PCR optimization for the transfer of markers between species of the *Melaleuca* genus. Scribner et al. (1996) provide examples of high levels of marker conservation in species ranging from whales to rodents to support their results in salmon and trout from Alaska, North America and the United Kingdom. What we have seen here, and in the previous study of abalone microsatellites (Huang & Hanna 1998) however, is a much lower rate of marker conservation between *Haliotis* species. This finding together with the very high levels of polymorphism encountered in most abalone species (*H. midae*, Evans et al. in prep. *H. asinina*, Selvamani et al. 2000: *H. rubra*, Evans et al. 2000), may point towards a more rapid mutation rate of microsatellite repeats in abalone than that seen in other organisms.

Research on the cross-amplification of microsatellite loci within taxa should ensure that the markers are useful within that species, and do not simply produce an amplification product. Our results have clearly shown that the presence of a similar sized PCR product on agarose gels is not sufficient to report locus conservation in another species. Future studies should therefore endeavor to test those products further by radioactive or fluorescent labeling methods similar to those that would be used in larger studies. This would ensure that only those markers that are likely to provide reliable genetic information would be considered by those commencing projects on these species.

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INDUCTION OF TRIPLOIDY IN PACIFIC RED ABALONE (*HALIOTIS RUFESCENS*)

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ABSTRACT Induction of triploidy was evaluated in red abalone, *Haliotis rufescens*, using cytochalasin-B (CB). Three experiments were done at different times of the year and with different CB concentrations, but triploids were produced only in May and November. The largest percentages of triploids were obtained in the November induction (85%, 100%, 100%, for 0.5, 0.75, 1.0 mg/L CB, respectively), and poorer triploidy success was obtained in the May induction (3%, 11%, 26%, for 0.3, 0.5, 0.7 mg/L CB). When the induction was done in November CB concentration did not play a significant role in success of triploidy, although it did play a role on relative survival to trochophore, with the two highest concentrations resulting in lower survivals. For the May and September inductions, treatment with CB had a significant effect on survival, but this was independent of CB concentration. The results on triploidy success at each time of induction paralleled those for larval viability at each of those times of the year when evaluated in untreated control larvae, with the largest survival to trochophore (55%) and the lowest coefficient of variation (CV = 12%) for survival of untreated larvae produced in November. Untreated larvae produced in May had an intermediate survival (37%) and CV (57%), whereas that produced in September had the lowest survival to trochophore (1%) and the largest CV (143%).

KEY WORDS: red-abalone, *Haliotis rufescens*, triploid, egg quality, larval viability

INTRODUCTION

Pacific red abalone (*Haliotis rufescens*) is found from southern Oregon–northern California in the USA to the north of the Baja California Peninsula in Mexico (Cox 1962, Lindberg 1992). Aquaculture production of this species began during the last decade in Mexico, where the whole life cycle is managed under controlled conditions. The growth rate in this abalone is slow, reaching 120 mm to 160 mm in three to four years (Tegner & Butler 1985), and ways to improve production need to be defined. Triploidy, among other methods, has been proposed to improve production of abalone (Fujino 1992, Elliott 2000).

Triploidy has been induced in a wide variety of marine mollusks (Beaumont & Fairbrother 1991), with the pioneer work having been on oysters (Stanley et al. 1981). For most species, induction of triploidy in mollusks is done by inhibiting the extrusion of the second polar body during the conclusion of meiosis in fertilized eggs. Several induction methods have been tested; chemical treatment with cytochalasin-B (CB) or 6-dimethylaminopurine (6-DMAP), and physical treatment with hydrostatic pressure or thermal shock (cold or heat). In abalone species, Arai et al. (1986) and Kudo et al. (1991) were the first to report successful production of triploid abalone (*Haliotis discus hannai* and *Haliotis diversicolor diversicolor*) using thermal shock. Curatolo and Wilkins (1995) produced triploids of *Haliotis discus hannai* using hydrostatic pressure. Recently, Zhang et al. (1998) produced triploids of that same species using 6-DMAP, Yang et al. (1998a), Yang et al. (1998b) produced triploids of *Haliotis diversicolor* using thermal shock and cytochalasin-B, and Stepto and Cook (1998) produced triploids of *Haliotis midae* using cytochalasin-B. Powers et al. (1996) mentioned their success in producing and growing triploids of Pacific red abalone, *Haliotis rufescens*, although no details are given in the abstract.

In this study we report on the effects of induction to triploidy using different CB concentrations in east Pacific red abalone, *Haliotis rufescens*, during three times of the year.

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MATERIALS AND METHODS

Three experimental inductions to triploidy were done at different times during the year 2000 in a private hatchery; late May, early September, and late November.

Spawning

Spawning and gamete collection procedures were the same for all experimental inductions. Mature spawners (10–15 cm shell length) of red abalone, *Haliotis rufescens*, were selected from the broodstock kept in the commercial farm 'Abulones Cultivados' at Erendira, Baja California, Mexico, and used to obtain eggs for the treatments. Spawning was induced following methods developed by Morse et al. (1977), by adding hydrogen peroxide dissolved (final concentration 0.02%) in seawater for a period of approximately two hours, or until spawning began.

Gamete Collection

Four sets or blocks were used for each of the three experimental induction times. Because the number of eggs shed by red abalone is not large enough to be able to divide the eggs from one female into different CB treatment concentrations, each set was composed of a mixture of eggs from 3–5 females. The sets were made sequentially, that is, eggs of the first three to five females that spawned were mixed to make the first set, and the other sets were made sequentially, each of them with 3–5 spawners. Eggs were fertilized by adding about 30 sperm per egg.

Triploid Induction

For each of the three experimental induction times, each set was divided into the same four treatment groups (one untreated control, and three CB treatment concentrations). For the May induction, the CB concentrations tested were 0.3, 0.5, and 0.7 mg L^{-1}; for the September induction 0.6, 0.8, and 1.0 mg of CB L^{-1} were tested; and for the November inductions the CB concentrations tested were 0.5, 0.75, and 1 mg L^{-1}. During the May induction, cytochalasin-B was added at the time at which the eggs in the

control group showed the beginning of the extrusion of the second polar body (PB2) and ended when 50% of the control group eggs showed the second polar body. On average, the treatment lasted 20 minutes. Water temperature during the induction was 15°C. Egg density for each treatment was 2×10^5 L^{-1}. For the inductions in September and November, CB was added when 50% of the eggs in control group showed the first polar body (PB1), and ended when 50% had the second polar body. For the September induction, treatment lasted from 19 to 55 minutes, depending on when 50% of the eggs in each set showed extrusion of PB2. Water temperature during treatments was 20°C, and egg density for all treatments was 1.2×10^5 L^{-1}. During the November induction, treatment lasted between 32 to 48 minutes, depending on the set. Water temperature during the induction was 14.5° C, and egg density for the treatments was 2.3×10^5 L^{-1}. For all three experimental induction times, after treatment with the different concentrations of CB, eggs were rinsed in DMSO (1 mL L^{-1}) for 15 minutes to remove any remaining CB (Allen et al. 1989).

Absolute and relative survival from egg to trochophore larvae (about 20 hours post fertilization) and absolute and relative survival from egg to postlarvae (about 120 hours post fertilization) were estimated for each treatment group and control for all experimental inductions. Relative survivals were estimated within each set and induction time as the ratio between the survival of the treated group to the survival in its control group, and expressed in percentages.

Ploidy success was estimated by flow cytometry (Ploidy Analyzer II, PARTEC Germany) of postlarval stages (approximately 200 / treatment / block), with the exception of the experimental induction in September, for which trochophore larvae (for those sets with surviving larvae) were analyzed. The flow cytometry methodology was that originally developed by Allen (1983), with modifications for larvae in Allen & Bushek (1992). In short, larvae were concentrated in a 1-ml suspension, centrifuged, and the pellet was stained with 0.5 ml of a DAPI/detergent/DMSO solution (Allen & Bushek 1992), re-suspending by vortex. Trochophore and postlarvae were disaggregated as in Allen and Bushek (1992); trochophore larvae by repeated aspiration with a 1 ml syringe, and postlarvae were disaggregated by means of crushing the meat/shell with a glass rod. In both cases, the obtained cell suspensions were passed through a 30-μm screen, adding 1.5 ml of the DAPI/detergent/DMSO solution before flow cytometry assay.

Larval Culture

After treatment, the embryos were kept in 100-L tanks until hatching. No feeding, aereation, or water flow was provided during this period (20 h). After hatching, swimming trochophore larvae was transferred to 150-L tanks at a stocking density of 4 larvae / ml, providing continuous flow. Trochophore larvae were induced to settle after 6 days following Morse et al. (1979) methodology, modified by Searcy-Bernal and Anguiano-Beltran (1998) by adding GABA (gamma-aminobutyric acid) at a final concentration of 1.6 μM. After settling, postlarvae was grown in 200-L circular tanks (112 cm diameter, 28 cm height) by placing 30,000 postlarvae / tank, feeding by inoculating each tank with 3000 cells/cm^2 of the benthonic microalgae *Navicula incerta*.

Statistical Analyses

For each experimental induction, the effect of CB concentration on mean larval survival, and mean triploid success (n = 4), was analyzed using a randomized block design analysis of variance (Neter et al. 1985). Set (block) was considered a random effect. Percentage data were transformed to arcsine for analysis (Zar 1999). Significance was set as at $P < 0.05$ for all analyses. Tukey pairwise comparisons were used to test for differences between means (Neter et al. 1985).

To establish whether there were differences in larval viability between dates of spawning regardless of CB treatments, the effect of experimental induction time on absolute survival of the control, untreated groups, was analyzed with the same model above.

RESULTS

The results on survival to trochophore and to postlarvae, and on success in triploid production for each experimental induction time are in Table 1.

Triploidy

Triploid abalone were successfully produced only during the May and November inductions. During the September induction, no triploids were detected in any of the tested concentrations. For the May induction, the highest concentration (0.7 mg CB L^{-1}) gave the best success in triploid production (26%), whereas the lowest concentration (0.3 mg CB L^{-1}) resulted in the lowest success in triploids produced (3%) which was not significantly different ($P > 0.05$) from the control (0% triploids). The intermediate concentration (0.5 mg CB L^{-1}) resulted in 11% triploids, which was not significantly different from the results using the lowest or the highest concentrations (Table 1A). There were no triploids detected in the control groups of any of the sets. For the November induction, all tested concentrations resulted in high success in triploid induction (Table 1C), all being significantly different from the control group, but not from each other. Only one of the four sets in the 0.5 mg L^{-1} treatment had less (40%) than 100% triploids. Success in triploidy ranged from 85% for the lowest concentration (0.5 mg CB L^{-1}) to 100% for the intermediate (0.75 mg CB L^{-1}) and highest concentration (1 mg CB L^{-1}) tested.

Survival

Treated and Control Groups—Within Experimental Induction Times

For the May induction, there were no significant differences in absolute survival to trochophore or postlarvae between the treated groups and the control (Table 1A). However, there were significant differences in relative survival to trochophore ($P < 0.05$), with the control group having a survival larger than both the 0.3 and 0.5 mg CB L^{-1} treated groups, but not than the 0.7 mg L^{-1} treated group. There were no significant differences between treated and control group for relative survival to postlarvae.

For the September induction, absolute survival to trochophore larvae was not different between concentrations or the control, but relative survival was significantly larger ($P < 0.05$) for the control group than for any of the treated groups, which did not differ significantly from each other. There were no surviving postlarvae in any group (Table 1B).

For the November induction, there were no differences in absolute survival to trochophore larvae between control and CB treated groups, but there were differences ($P < 0.05$) in absolute survival to postlarvae, with the control group showing the largest survival, and the CB treated groups not being different from each

TABLE 1.

Absolute and relative mean survival to trochophore larvae and postlarvae treated with different concentrations of CB, and percent of triploidy success in Pacific red abalone, Haliotis rufescens, induced to triploidy in (A) May, (B) September, and (C) November. n = 4 for all induction times.

CB Treatment	Absolute		Relative		
	Survival to Trochophore (%)	Survival to Postlarvae (%)	Survival to Trochophore (%)	Survival to Postlarvae (%)	Percent Triploidy
(A)					
Control	37^{a}	10^{a}	100^{a}	100^{a}	0^{a}
0.3 mg L^{-1}	20^{a}	5^{a}	56^{b}	54^{a}	3^{a}
0.5 mg L^{-1}	21^{a}	5^{a}	58^{b}	50^{a}	11^{ab}
0.7 mg L^{-1}	23^{a}	2^{a}	63^{ab}	24^{a}	26^{b}
(B)					
Control	0.96^{a}	0	100^{a}	0	0^{a}
0.6 mg L^{-1}	0.44^{a}	0	20^{b}	0	0^{a}
0.8 mg L^{-1}	0.46^{a}	0	34^{b}	0	0^{a}
1.0 mg L^{-1}	0.30^{a}	0	10^{b}	0	0^{a}
(C)					
Control	55^{a}	46^{a}	100^{a}	100^{a}	0^{a}
0.5 mg L^{-1}	48^{a}	23^{b}	84^{ab}	47^{b}	85^{b}
0.75 mg L^{-1}	31^{a}	12^{b}	56^{b}	24^{b}	100^{b}
1.0 mg L^{-1}	32^{a}	13^{b}	60^{b}	27^{b}	100^{b}

Within columns and experimental induction time, different letters are used when means are significantly different (ANOVA, $P < 0.05$).

other. Relative survival to trochophore larvae and to postlarvae was also different from the control group. The lowest relative survival to trochophore was seen for the two highest tested CB concentrations (0.75 and 1 mg L^{-1}), whereas the lowest CB concentration did not show significant differences with the control or the other tested CB concentrations. All treated groups had significantly lower relative survival to postlarvae than the control group (Table 1C).

Control Groups—Between Experimental Induction Times

Survival of trochophore and postlarvae in the control groups was significantly different ($P < 0.05$) between experimental induction times (Table 2). The highest survival was seen for larvae produced in November (55% to trochophore larvae, 46% to postlarvae), followed by larvae produced in May (37% to trochophore larvae, 10% to postlarvae). For the September spawn, there was only 1% survival to trochophore larvae, and no surviving postlarvae. The coefficients of variation for mean survival was high for the September induction, followed by May, and with the lowest CV the survivals in November.

TABLE 2.

Absolute means survival (%) and coefficient of variation (CV) from egg to trochophore and from egg to postlarvae of the control untreated groups, for each of the experimental inductions.

Induction time	Survival Mean % (CV) Egg to Trochophore	Survival Mean % (CV) Egg to Postlarvae
May	36.7^{b} (57)	9.6^{a} (62)
September	1.0^{a} (143)	—
November	55.3^{c} (12)	46.0^{b} (28)

Different letters within columns indicate significant differences between induction times.

DISCUSSION

Our results show that triploidy can be induced in red abalone, *Haliotis rufescens*, using cytochalasin-B (CB) to inhibit release of the second polar body, and that factors other than CB concentration can have an effect on success of triploid induction as evidenced by the different results obtained between May and November in spite of having used two equal CB concentrations. The cause for the variable success rate in triploids produced between the three experimental induction times is not known, but three possible causes might have been influencing the results, either independently or combined: different temperatures during the spawning and inductions, differences in the CB concentrations and duration of the inductions, and differences in egg quality of abalones used in each of those months.

During the three induction times the temperature was different only for the September induction, when a unusually high temperature of 20°C was recorded and a low survival to trochophore with no triploids produced, and no surviving postlarvae were observed. The effect of increased water temperature on larval viability during the spawning for this species is not known, and no inferences can be drawn with regard to this factor alone. Furthermore, in May and November the results on triploidy success were different between both inductions, but water temperature was about the same for both inductions, 14.5°C and 15°C. Whereas it is known that at least in oysters meiotic rates are increased as temperature increases (Downing & Allen 1987, Eudeline et al., 2000b), in our study meiotic rates were actually slower during the September induction (40 min for 50% PB1) than for the November induction (27 min for 50% PB1) in spite of the higher water temperature occurring during the September induction. This points toward another factor rather than temperature on itself being the cause of no success in triploid production during September. The second possible cause, CB concentration and duration of induction, can not explain completely the difference between induction times in September and

November because similar concentrations were tested, and in both cases the biological criteria defined by Allen and Bushek (1992) were used for the inductions; starting when 50% of the eggs showed the first polar body (PB), and ending when 50% showed the second PB. When the May and November induction results are compared, they indicated that CB concentration was important for the percentage of triploids produced only during the May induction but not during the November induction. However, this might be explained by differences in the duration of the induction between May and November, because induction in May was not started until the appearance in the first eggs of the extrusion of the second polar body, whereas in November it was started before, when 50% of the eggs had the first polar body. Given the shorter treatment duration in May resulting from this (20 min) when compared with the other induction times (>30 min), increasing CB concentrations might have been important in inducing a larger percentage of triploids during the short induction time in May. Supan et al. (2000) found that whereas increasing CB concentration was important for improved success in triploidy induction for the American oyster, *Crassostrea virginica*, a longer treatment time might have resulted in greater triploidy success. They used a fixed treatment time (10 min), which as in our case for the May induction, did not follow completely the biological criteria recommended by Allen and Bushek (1992) for triploid induction.

The third possible cause for the differences in triploidy success between induction times could have been differences in reproductive condition of the abalones between May, September, and November. There are few studies on the reproductive cycle of red abalone native to the study area. From the two found it is known that the reproductive cycle of red abalone in northern Baja California is active from May to December, reaching maturity around September (Ortiz-Quintanilla et al. 1990), with the spawning peak occurring between October and December (Molina-Martínez 1983). If during the year of the experiment the reproductive cycle followed that described, mature individuals should have been available at all three induction times, and in fact, they were. However, an observation during the induction times was that, even if available, mature spawners were more difficult to find at the farm during the May induction, and still more difficult to find in September than in November, when the largest success was seen in triploid production. Then, whereas the variable abundance of mature spawners appears to indicate that the egg quality was variable between those induction times, biochemical determination of egg quality was not done. However, alternative or indirect measurements of egg quality that do not involve biochemical determinations of reserves have been previously used in association with evaluations of triploid success. For example, Aldridge et al. (1990) used survival in the control groups as a measurement of egg quality, finding an association between survival in the controls and success in triploid induction in bighead carp. They proposed that egg quality, measured as larval viability in the control groups, could provide a good indicator of success in triploid production. In the present study survival in the control groups indicated also an association with triploidy success, with the highest survivals to trochophore larvae having seen during November, when the largest success to triploidy was obtained, and the lowest triploidy success was observed when the lowest survival occurred in the control groups. Furthermore, the coefficients of variation (CV) for mean survival to trochophore larvae in the control groups for each induction time, with the lowest CV seen for the November data, and the highest CV for the September data, are also indicative of large variability in survival being associated to low success in triploid production and low variability in survival with high success. The importance of evaluating survival in the control groups rather than in the treated groups as a potential indicator of success in triploidy is demonstrated by the fact that Allen and Bushek (1992) did not find a correlation between survival of treated batches or families of oyster eggs and success in triploidy, nor did Ruiz-Verdugo et al. (2001) when working with scallop eggs. However, the last authors found that the correlation between number of eggs and number of D-larvae in untreated groups was highly significant, whereas in the CB treated groups that correlation was lost, indicating that survival in treated groups is affected by factors other than egg quality, as for example, toxicity of the CB treatment itself.

The importance of egg quality in triploid induction of mollusks have been stressed by Utting and Doyou (1992), who demonstrated that variability in egg quality among the treated eggs of the Manila clam, *Ruditapes philippinarum*, can affect success during triploid induction. This is presumably caused by a lack of synchrony in meiotic rates, which are known to be highly important in triploid and tetraploid induction (Allen & Bushek 1992, Eudeline et al. 2000a). In the present study, there was evidence of lower synchronization in meiotic rates between the sets used during September than those used during November. That evidence comes from the coefficient of variation (CV) of meiotic rate between sets, measured as time to reach 50% of PB1, which was twice during the September induction (CV = 33%) than in November (CV = 16%).

In conclusion, success of triploidy in Pacific red abalone can be obtained when using CB concentrations from 0.5 to 1 mg L^{-1}, and when the induction is started when 50% of the eggs show extrusion of the first polar body. Larger concentrations of CB will result in lower survival of treated eggs, possibly as an effect of toxicity of the chemical. Variation among induced batches at different times of the year can be expected, and this might be a consequence of differences in the egg quality of the spawners at each time, although this has to be further evaluated using biochemical data.

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THE EFFECTS OF ENVIRONMENTAL FACTORS ON THE BIOCHEMICAL COMPOSITION OF THE BIVALVE *TAGELUS DOMBEII* (LAMARCK, 1818) (TELLINACEA: SOLECURTIDAE) FROM THE INTERTIDAL FLAT OF COIHUÍN, PUERTO MONTT, CHILE

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ABSTRACT Samples of *Tagelus dombeii* (eight adults of each sex ranging between 5.5 cm and 7.0 cm) were collected monthly from the tidal flat of Coihuin, Puerto Montt, Chile, between June 1995 and July 1996. The soft tissue was divided into gonad, digestive gland and remaining somatic tissue, which were dried before determination of dry weight and biochemical composition (protein, lipid, carbohydrate and ash). Several environmental variables were measured at the same time (temperature, salinity, seston, clorophyll *a*, phytoplankton). Data were standardized to one individual of 6.0 cm shell length and presented as monthly means ± s.d. for each sex.

There was no seasonal pattern in the dry meat weight of *T. dombeii*. The spring diatom bloom of 1995 was not reflected in an increase in dry body weight. The increases in the weights of the somatic fraction and the digestive gland during the winter of 1996, together with increases in all the biochemical components, were attributable to an unusual diatom bloom and a reduced metabolic expenditure associated with lower temperatures. The cycle in the biochemical components is reflected in the energy content, which follows the trend in the weight of the corresponding tissue.

There is an increase in the gonad weight and in the gonad index in *T. dombeii* in spring and a further increase in summer. Gametogenesis occurs initially at the expense of reserves accumulated earlier in the soma (principally carbohydrate and protein) or in the digestive gland (mostly lipid), resulting in decreases in the storage tissues as the reserves are transferred to the gonad. Subsequently, energy for gametogenesis is obtained directly from ingested food as this source becomes adequate. Nevertheless, whether gametogenesis is associated with high body condition or with an abundant food source, there is no decrease in the weight of the animal.

The annual variation in the biochemical components of the tissues is closely coupled to the availability of food, to temperature cycles that regulate metabolism and to the processes of gamete synthesis and release. Temporal fluctuations in environmental factors, especially temperature and food supply, drive the cycles of storage and utilization of metabolic energy reserves, which in turn govern growth and gametogenesis in this species.

KEY WORDS: bivalves, *Tagelus dombeii*, environmental variables, biochemical composition

INTRODUCTION

The annual and interannual variation in the availability of nutrients, in both qualitative and quantitative terms, seems to determine intraspecific variation in growth, reproduction and energy storage in bivalves (Newell et al. 1982, MacDonald & Thompson 1985a, MacDonald & Thompson 1985b, MacDonald & Thompson 1986, Emmett et al. 1987). In general, the synthesis and storage of protein, carbohydrate and lipid in molluscs occurs during those months in which food availability is greatest (Pieters et al. 1980, Ruiz et al. 1992, Hawkins et al. 1985, Emmett et al. 1987), these reserves being utilized when food becomes scarcer (Ansell 1974) or when metabolic demands such as those of gametogenesis must be met (Gabbott & Bayne 1973, Bayne 1976, Soniat et al. 1984, Soniat & Ray 1985, Emmett et al. 1987).

Depending on the availability of food and the size of the individual, energy may be allocated to the synthesis of body tissue, i.e., growth, or to the production. Ansell and Trevallion (1967) found that in *Tellina tenuis* dry body weight can increase by 44% when food is abundant and the gonad is developing, and decreases sharply during spawning. When the decrease in the weight of one or more tissues coincides with gonad development, one may conclude that the reproductive process is at least partly dependent on accumulated energy reserves. The weights of the digestive gland and adductor muscle decrease as gonad weight increases in *Patinopecten yessoensis* (Fuji & Hashizume 1974) and *Placopecten magellanicus* (Robinson et al. 1981). Thus reproduction is a process which requires a considerable expenditure of energy, obtained from recently ingested food or from the catabolism of reserves in the body tissues (Gabbott 1976, Gabbott 1983). In some species, such as *Pecten maximus* (Comely 1974) and *Chlamys opercularis* (Taylor & Venn 1979), energy reserves are laid down in summer for utilization during gametogenesis in the autumn, whereas in others, e.g., *Chlamys septemradiata* (Ansell 1974) and *Placopecten magellanicus* (Thompson 1977), the maximum food supply coincides with gonad growth and development. There are also species which can adopt either strategy, depending on the population (Thompson 1984, Gabbott 1983), or which utilize both strategies, depending on the time of year. Consequently, the seasonal variation in the biochemical composition of bivalve tissues is the result of complex interactions between environmental factors and metabolic processes (Gabbott 1975, Gabbott 1976, Gabbott 1983, Beninger & Lucas 1984, Thompson & MacDonald 1990), which are reflected in fluctuations in the dry weight of the different body tissues (Ansell 1972, Barber & Blake 1981, Shafee 1981).

In cold temperate environments the food supply to bivalves and physical factors exhibit considerable spatial and temporal variation (Vahl 1980, Dellarosa et al. 1993, Navarro et al. 1993, Stead et al. 1997, Toro et al. 1999). Navarro and Jaramillo (1994) concluded that the principal contribution of phytoplankton to the nutrition of suspension-feeders in the south of Chile, occurs during spring and summer, values being minimal during the rest of the year. Consequently, *Venus antiqua* exhibits a markedly seasonal growth pattern with high rates of growth in spring and summer (Clasing et al. 1994). Since the patterns of growth and reproduction in bivalves reflect environmental conditions (Trevallion 1971, Ineze et al. 1980, Chaparro & Winter 1983), it is likely that temporal changes in temperature and food supply are important in the regulation of

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biochemical cycles and reproductive processes. According to Shafee (1981) and Thompson and MacDonald (1990), these environmental variables can affect the biochemical components of the gonads of males and females in different ways, owing to the differences in the biochemical composition of the gametes.

The objective of the present study was to characterize and quantify the temporal variation in the biochemical composition (lipid, carbohydrate, protein and inorganic material) and weights of various body tissues in the bivalve *Tagelus dombeii* (Lamarck 1818), and to establish their relationships with several environmental variables (temperature, salinity, seston, pigments, phytoplankton) over the intertidal flat of Coihuín, Puerto Montt, Chile.

MATERIALS AND METHODS

Samples of *T. dombeii* were collected monthly between June 1995 and July 1996 from the intertidal flat of Coihuín (41°29′S; 72°54′W), located approximately 8 km SE from the town of Puerto Montt (Fig. 1). At each sampling time, water temperature and salinity were recorded and water samples (3 replicates of 2 liters volume) collected for determination of chlorophyll *a*, phaeopigments, particulate organic matter and particulate inorganic matter (Strickland & Parsons 1972). Additional water samples were fixed in Lugol's iodine for quantification of the principal phytoplankton taxa (diatoms and microflagellates).

For determination of tissue dry weight and subsequent biochemical analyses, 8 individual clams of each sex (determined by microscopic inspection of gonad smears), ranging between 5.5 cm and 7.0 cm valve length, were opened and the soft tissues separated into digestive gland, gonad and the rest of the tissue (soma). Separated tissues were dried at 60°C for 48 hours, weighed, ground in a ball mill (Retschmühle type MM) and stored at −20°C for biochemical analysis. Gonad and digestive gland indices were calculated as the dry weight of each tissue as the percentage of the total dry weight of soft tissues.

Carbohydrate, lipid, protein and ash were determined on subsamples of dried, homogenized tissue. Carbohydrate was estimated by the phenol-sulphuric acid procedure (Dubois et al. 1956) and lipid gravimetrically according to Bligh and Dyer (1959). Total nitrogen was determined with a CHN analyser (Perkin Elmer 2400) standardized with acetanilide, and protein calculated as 5.8 × N (Gnaiger & Bitterlich 1984). Ash-free dry weight was determined by combustion of dry samples for 12h at 500°C. Biochemical data were expressed as absolute values per standardized individual clam.

To express the biochemical data in terms of energy, values for the different tissues were multiplied by the appropriate conversion factors (protein: 24 $KJ \cdot g^{-1}$, lipid: 39.5 $KJ \cdot g^{-1}$, carbohydrate: 17.5 $KJ \cdot g^{-1}$, Gnaiger 1983).

Data for the dry weight, biochemical composition and energy content of the tissues were presented for males and females separately as monthly means with standard deviations. Values were corrected for a "standard" clam of shell length 6 cm, as described by Bayne et al. (1987):

$$W_S = (L_S/L_L)^b \times W_E$$

Where: W_S is the dry weight of the standard clam, L_S the standard shell length (6 cm), L_L and W_L the recorded shell length and dry weight respectively of the experimental clam, and b the weight exponent of the relationship between length and weight.

Student's *t*-test was used to test for differences between mean values for males and females. The relationships between dry weight, biochemical composition, food availability and physical factors were examined by correlation analysis and principal components analysis (Statistica 4.2 for Windows).

RESULTS

Environmental Factors

A seasonal cycle in water temperature was observed, with minimum values in winter (9°C in July 1995, 10°C in July 1996), increasing gradually until summer (17°C in January 1996). Salinity remained stable, varying between 24.9‰ in December 1995 and 32.4‰ in May 1996 (Fig. 2a).

Total particulate matter (seston) was highest in autumn-winter, reaching 10 $mg \cdot l^{-1}$ in July 1995 and 12 $mg \cdot l^{-1}$ in July 1996. During spring and summer values remained around 5 $mg \cdot l^{-1}$. The organic fraction of the seston exhibited a similar cycle (Fig. 2b), with minimum values (0.63 $mg \cdot l^{-1}$) in September 1995 and maximum values (5.57 $mg \cdot l^{-1}$) in June of the same year.

The concentration of chlorophyll *a* was lowest in summer (0.36 $\mu g \cdot l^{-1}$) and highest in April and May of 1996 (7.57 $\mu g \cdot l^{-1}$ and 8.61 $\mu g \cdot l^{-1}$ respectively). There were two minor peaks in July and September 1995 (Fig. 2c). Phaeopigment concentrations were lower than 2 $\mu g \cdot l^{-1}$ throughout 1995. In April 1996 a value of 7.8 $\mu g \cdot l^{-1}$ was recorded, probably as result of high zooplankton activity. Diatoms showed two main peaks (Fig. 2d), the first in November 1995 (5129 $cells \cdot ml^{-1}$) and the second in April and May 1996 (6954 $cells \cdot ml^{-1}$ and 5626 $cells \cdot ml^{-1}$ respectively), whereas high densities of microflagellates were observed only in the summer 1995–1996 (1833 $cells \cdot ml^{-1}$).

Dry Tissue Weight

Somatic Tissue

No large variations in the dry weight of somatic tissue of T. *dombeii* were observed during the study, although values were

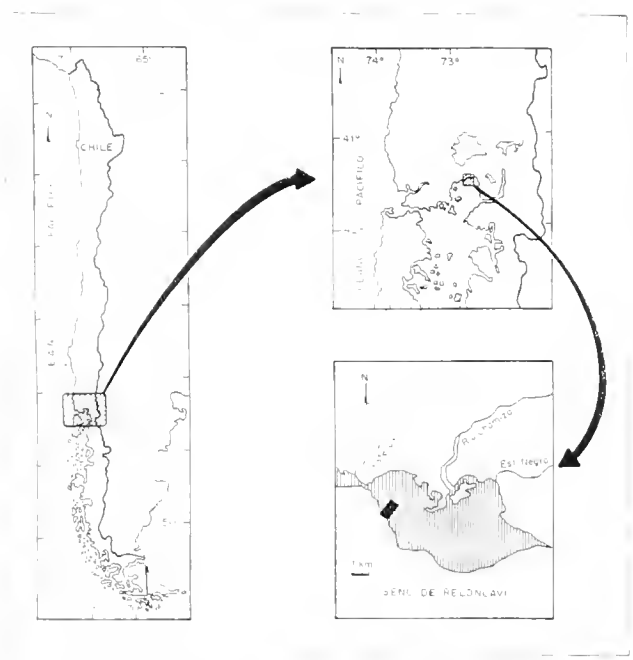

Figure 1. Map of the tidal flat at Coihuín, Puerto Montt, Chile, showing the location of the sampling area.

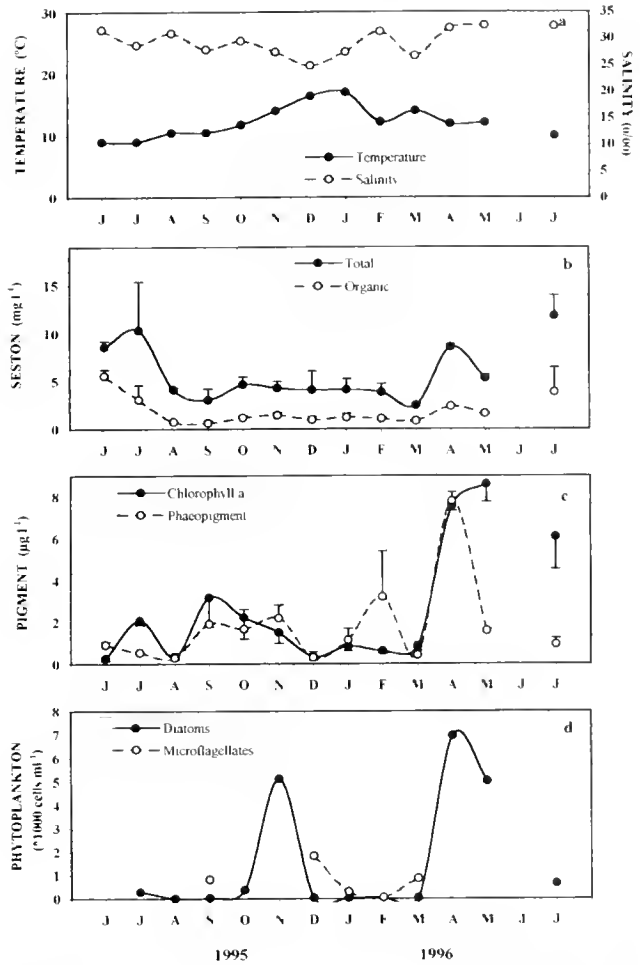

Figure 2. Temporal variation of the temperature and salinity (a) total and organic seston; (b) chlorophyll *a* and phaeopigments; (c) diatoms and microflagellates; (d) present in the water column. Values are, means ±s.d.

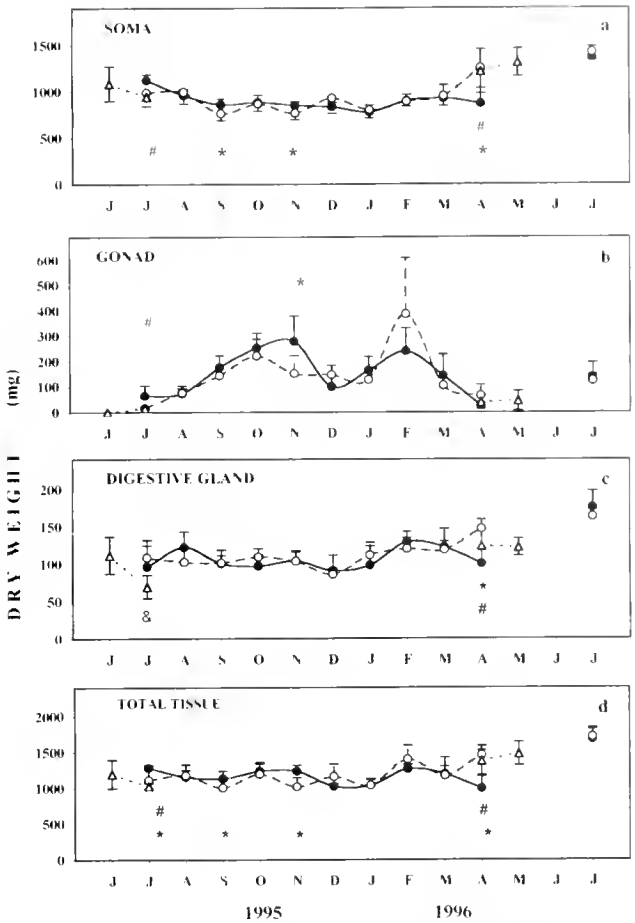

Figure 3. *T. dombeii*. Temporal variation in the dry weights of somatic tissue (a) gonad; (b) digestive gland; (c) and total tissue; (d) to one individual of 6.0-cm shell length. Data presented as monthly means ± s.d. of males (●), females (○) and indeterminates (△).
***: Significant differences between males and females at the $P \leq 0.05$ level**
#: Significant differences between males and indeterminates at the $P \leq 0.05$ level
&: Significant differences between females and indeterminates at the $P \leq 0.05$ level.

slightly higher during autumn and winter, especially during 1996 (Fig. 3a). With a few exceptions, males and females did not differ in somatic dry weight ($P \leq 0.05$). This observation was confirmed by the lack of a significant difference between sexes when the overall annual mean was compared.

In some clams it was not possible to determine the sex owing to the scarcity of gonad tissue. These were classified as "indeterminate", and constituted the entire sample in June 1995 and May 1996. When indeterminate individuals were present as well as males and females, their somatic weight was similar to that of the females but significantly different ($P \leq 0.05$) from that of the males (Fig. 3a).

Gonad

Gonad dry weight increased markedly during spring and summer (Fig. 3b). No significant differences were found between males, females and indeterminate individuals, except in July and November 1995 ($P \leq 0.05$). Changes in gonad weight suggests one spawning in spring and another in summer, as also demonstrated by the gonad index. No significant differences were observed between annual mean values for males and females, although indeterminate individuals exhibited much lower values ($P \leq 0.01$).

Digestive Gland

Values increased during 1996 to reach maximum values in July (Fig. 3c). Significant differences ($P \leq 0.05$) were observed between mean values for males and females only in July 1995 and April 1996. The annual means for digestive gland were similar in males, females and indeterminates (>0.05).

Total Weight

The variations in total dry weight were similar to those exhibited by somatic weight, because the latter represented the major component throughout the year (Fig. 3d). There were no significant differences ($P > 0.01$) in overall mean total dry weight between males, females and indeterminates.

Gonad and Digestive Gland Indices

Minimum values were observed for the gonad index at the beginning of the study (June 1995, Fig. 4a), i.e., all individuals were indeterminate. During the spring, gonad index increased to peak values of 22.3% in males (November) and 18.3% in females (October) before decreasing in December to 9.8% (males) and 12.8% (females). A second peak was observed in February of 18.9% for males and 26.5% in females, to decrease to minimum values of 2% in autumn (April). Only in November 1995 there was a significant difference ($P \leq 0.05$) in gonad index between sexes. In those months in which clams of indeterminate sex were observed (Fig. 4a), such individuals exhibited the lowest gonad indices, suggesting that at these times the gonad was regressing and/or that gametogenesis was about to begin.

The digestive gland index remained relatively constant throughout the year (Fig. 4b). Values varied between 7.5% and 10.6% in males and 7.5% and 10.8% in females. Comparisons of monthly means showed significant differences ($P \leq 0.05$) between males and females in 6 months (Fig. 4b), but there was no significant difference overall ($P > 0.05$) between males and females.

Proximate Biochemical Composition

Composition of Somatic Tissue

The protein content varied from 551 mg to 865 mg in males, from 521 mg to 871 mg in females, and from 623 to 868 mg in indeterminate individuals, with slightly higher values in winter of 1995 and autumn of 1996 (Fig. 5a). Significant differences were observed ($P \leq 0.05$) between males and females in September, November and December 1995 and in April 1996, and between

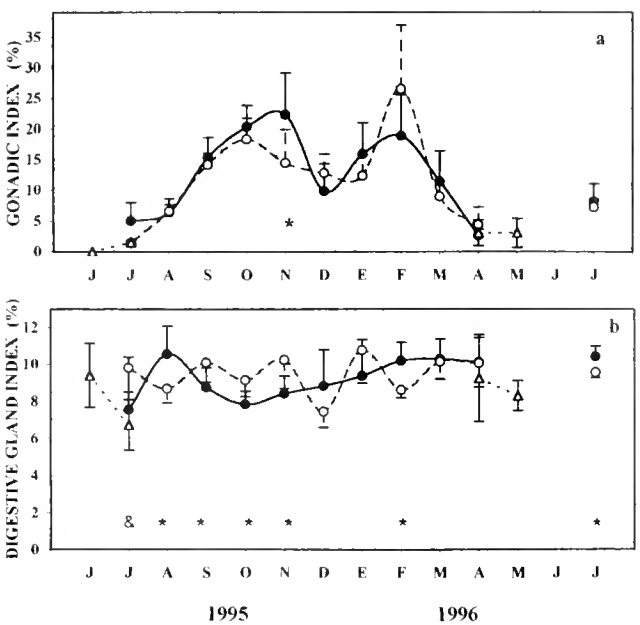

Figure 4. ***T. dombeii.*** **Temporal variation of the gonad index and the digestive gland index to one individual of 6.0-cm shell length. Data presented as, monthly means ± s.d. of males (●), females (○) and indeterminates (△).**
***: Significant differences between males and females at the $P \leq 0.05$ level**
&: Significant differences between females and indeterminates at the $P \leq 0.05$ level.

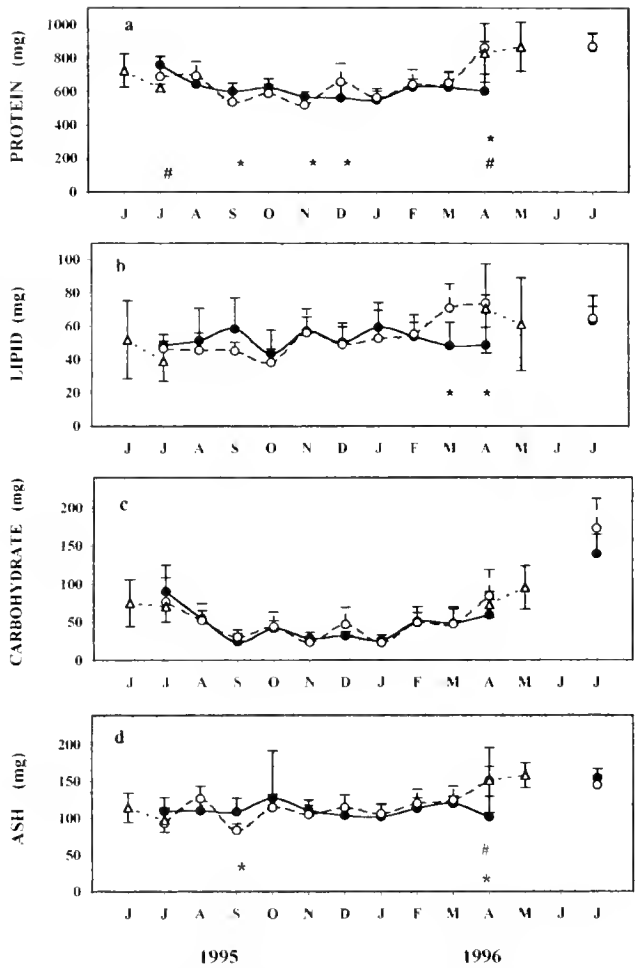

Figure 5. ***T. dombeii.*** **Temporal variation in the biochemical components of the somatic tissue, (a) Protein; (b) Lipid; (c) Carbohydrates; and (d) ash; to one individual of 6.0-cm shell length. Data presented as, monthly means ± s.d. of males (●), females (○) and indeterminates (△).**
***: Significant differences between males and females at the $P \leq 0.05$ level**
#: Significant differences between males and indeterminates at the $P \leq 0.05$ level.

males and intermediates in July 1995 and April 1996. There were no significant differences ($P > 0.05$) in annual means between males, females and indeterminate clams. Lipid content varied between 44 mg and 63 mg in males, 38 mg and 74 mg in females, and 39 and 71 mg in indeterminate clams (Fig. 5b). No significant differences ($P > 0.05$) were observed between males and females in the annual means, although values were significantly different ($P \leq 0.05$) in March and April 1996. Carbohydrate content was lowest in spring-summer and highest in autumn-winter (Fig. 5c), reaching a maximum in July 1996. Intermediate values were observed in indeterminate individuals. Significant differences were only observed ($P \leq 0.01$) between annual means for indeterminate clams and those for males or females. Ash content varied between 101 mg and 154 mg in males and 84 mg and 151 mg in females, with means of 115 ± 28 mg, n = 83 and 114 ± 23 mg, n = 81, respectively (NSD, $P > 0.05$). Significant differences were observed ($P \leq 0.05$) between males and females in September 1995 and between males and females and males and indeterminate clams in April 1996 (Fig. 5d). The annual mean ash weight in

indeterminate clams was significantly different from that for males ($P \leq 0.01$) and females ($P \leq 0.05$).

Composition of the Gonad

In some months (June and July 1995, April and May 1996) insufficient tissue was available to undertake the biochemical analyses (indicated by "?" symbol, Fig. 6).

Protein content showed two rises (Fig. 6a): the first in October 1995 with the highest value for males (186 mg) and the second in February 1996 with the highest value for the females (221 mg). The lowest protein content was observed in August 1995 for both sexes (48 mg in males and 51 mg in females). Values for both sexes from September to November 1995 and from January and March 1996 were significantly different ($P \leq 0.05$). The average annual protein level in males was significantly different ($P \leq 0.01$) from that obtained for females. Lipids were notoriously higher in October and in February (Fig. 6b), at the time when gonad weight showed the highest values (Fig. 3b). There were no significant differences ($P \leq 0.05$) between the monthly averages nor in annual averages obtained for this component. The carbohydrates showed a similar tendency with marked increases in October and a maximum value in the case of the females in February. Figure 6c shows the months in which the monthly averages for both sexes were significantly different ($P \leq 0.05$). No significant differences were observed when annual averages were compared ($P > 0.05$). Ash content (Fig. 6d) increased significantly in November 1995 and in February 1996. Significant difference between males and females was only observed in July 1996. No significant differences were observed when compared annual means ($P > 0.05$).

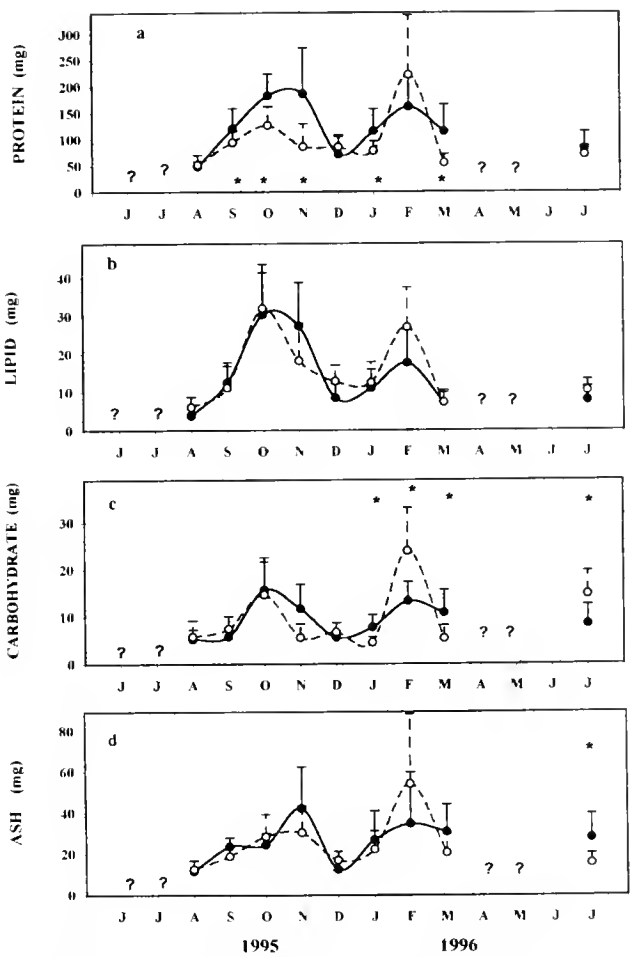

Figure 6. *T. dombeii.* **Temporal variation in the biochemical components of the gonad, (a) Protein; (b) Lipid; (c) Carbohydrates; and (d) ash; to one individual of 6.0-cm shell length. Data presented as, monthly means ± s.d. of males (●), females (○).**
***: Significant differences between males and females at the $P \leq 0.05$ level**
?: without information by tissue scarcity.

Composition of the Digestive Gland

Protein was highest in July 1996 with a minimum value found in July 1995 in males and in December in the females; in indeterminate individuals protein fluctuated between 30 mg in July 1995 and 57 mg in April 1996 (Fig. 7a). There were significant differences ($P \leq 0.05$) between males and females in August 1995 and

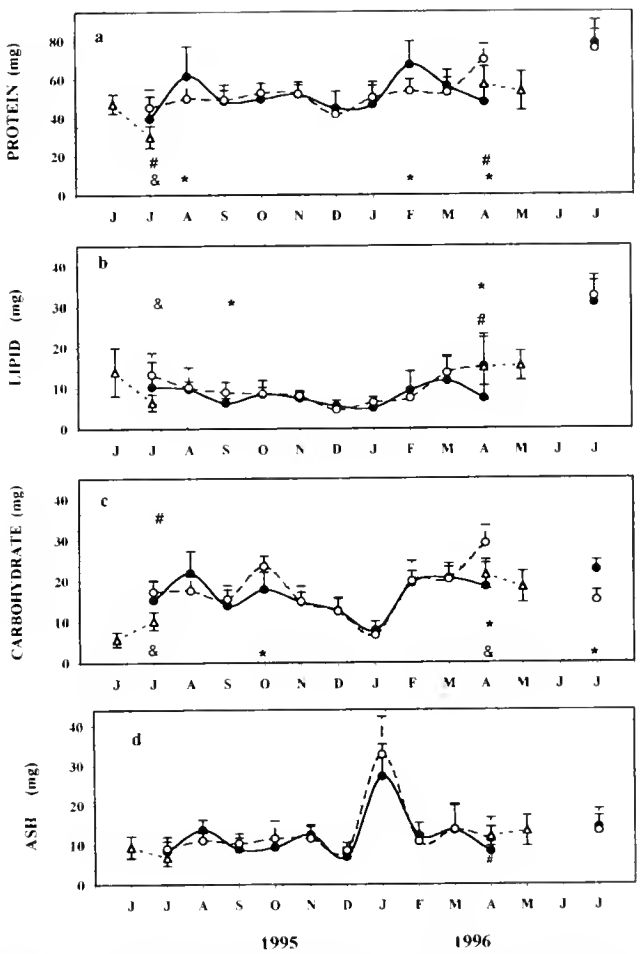

Figure 7. *T. dombeii.* **Temporal variation in the biochemical components of the digestive gland, (a) Protein; (b) Lipid; (c) Carbohydrates; and (d) ash; to one individual of 6.0-cm shell length. Data presented as monthly means ± s.d. of males (●), females (○) and indeterminates (△).**
***: Significant differences between males and females at the $P \leq 0.05$ level**
#: Significant differences between males and indeterminates at the $P \leq 0.05$ level
&: Significant differences between females and indeterminates at the $P \leq 0.05$ level.

TABLE 1.

Tagelus dombeii. Pearson correlation coefficients between environmental and body components during two relevant periods of the life cycle.

Winter n = 49	T°	Chl a	TS	OS	Phyt	STW	GTW	DGTW	GI	S Prot	S Lip	S Cho	G Prot	G Lip	G Cho	DG Prot	DG Lip	DG Cho
T°	1,00	−0,09	**−0,99**	**−1,00**	0,25	**−0,45**	**0,70**	**0,29**	**0,73**	**−0,35**	0,21	**−0,64**	–	–	–	**0,50**	−0,19	0,27
Chl a		1,00	−0,03	0,06	**0,94**	**−0,44**	**0,45**	−0,25	**0,50**	**−0,37**	0,07	**−0,28**	**0,76**	**0,66**	0,24	−0,24	−0,20	**−0,45**
TS			1,00	**1,00**	**−0,37**	**0,50**	**−0,76**	−0,27	**−0,80**	**0,39**	−0,22	**0,68**	**−0,76**	**−0,66**	−0,24	**−0,47**	0,22	−0,21
OS				1,00	**−0,29**	**0,47**	**−0,72**	**−0,29**	**−0,75**	**0,36**	−0,21	**0,66**	**−0,76**	**−0,66**	−0,24	−0,49	0,20	−0,25
Phyt					1,00	**−0,58**	**0,68**	−0,14	**0,74**	**−0,48**	0,14	**−0,50**	**0,76**	**0,66**	0,24	−0,06	−0,26	**−0,35**
STW						1,00	**−0,40**	0,19	**−0,53**	**0,88**	0,14	**0,77**	−0,33	−0,33	−0,01	0,07	**0,41**	0,22
GTW							1,00	0,16	**0,98**	−0,26	**0,33**	**−0,59**	**0,99**	**0,79**	**0,55**	**0,30**	−0,20	0,03
DGTW								1,00	0,10	0,18	0,26	0,03	−0,13	−0,26	0,02	**0,85**	**0,58**	**0,88**
GI									1,00	**−0,38**	0,26	**−0,66**	**0,95**	**0,78**	**0,49**	0,25	−0,24	−0,03
S Prot											0,14	**0,58**	−0,21	−0,23	0,02	0,10	**0,41**	0,19
S Lip											1,00	−0,03	0,21	0,18	0,19	**0,34**	0,12	0,18
S Cho												1,00	**−0,55**	**−0,40**	0,04	−0,10	**0,45**	0,09
G Prot													1,00	**0,78**	**0,50**	−0,07	−0,29	−0,31
G Lip														1,00	**0,60**	−0,21	**−0,43**	**−0,37**
G Cho															1,00	0,07	0,02	0,01
DG Prot																1,00	**0,44**	**0,77**
DG Lip																	1,00	**0,50**
DG Cho																		1,00

Spring Summer n = 46	T°	Chl a	TS	OS	Phyt	STW	GTW	DGTW	GI	S Prot	S Lip	S Cho	G Prot	G Lip	G Cho	DG Prot	DG Lip	DG Cho
T°	1,00	**−0,81**	**−0,96**	**−0,75**	**−0,99**	0,04	**−0,44**	−0,11	**−0,40**	0,17	−0,09	0,12	**−0,37**	**−0,58**	**−0,32**	**−0,33**	**−0,58**	**−0,68**
Chl a		1,00	**0,94**	**1,00**	**0,74**	−0,27	**0,46**	**0,33**	**0,47**	**−0,30**	0,20	**−0,40**	**0,41**	**0,55**	0,28	**0,46**	**0,63**	**0,32**
TS			1,00	**0,90**	**0,92**	−0,15	**0,47**	0,22	**0,45**	−0,24	0,15	−0,26	**0,41**	**0,60**	**0,32**	**0,41**	**0,63**	**0,55**
OS				1,00	**0,66**	**−0,30**	**0,45**	**0,35**	**0,46**	**−0,31**	0,21	**−0,43**	**0,40**	**0,53**	0,27	**0,47**	**0,61**	0,25
Phyt					1,00	0,01	**0,42**	0,06	**0,36**	−0,13	0,06	−0,06	**0,35**	**0,56**	**0,31**	0,28	**0,54**	**0,73**
STW						1,00	−0,02	0,00	−0,21	**0,90**	0,15	**0,65**	−0,04	−0,06	0,12	0,01	−0,09	0,10
GTW							1,00	0,08	0,97	−0,06	−0,04	−0,06	**0,98**	**0,90**	**0,89**	0,24	0,24	0,28
DGTW								1,00	0,03	0,06	0,16	−0,12	0,06	0,12	0,06	**0,93**	**0,64**	0,20
GI									1,00	−0,23	−0,06	−0,18	**0,96**	**0,88**	**0,85**	0,18	0,18	0,22
S Prot										1,00	0,10	**0,54**	−0,09	−0,08	0,02	0,03	−0,07	−0,04
S Lip											1,00	0,03	−0,05	−0,03	0,00	0,17	−0,04	−0,10
S Cho												1,00	−0,08	−0,15	0,07	−0,12	−0,25	0,26
G Prot													1,00	0,86	**0,89**	0,22	0,18	0,22
G Lip														1,00	**0,73**	0,29	**0,34**	**0,36**
G Cho															1,00	0,22	0,12	0,27
DG Prot																1,00	**0,74**	**0,33**
DG Lip																	1,00	**0,39**
DG Cho																		1,00

T°: Temperature; Chl a: chlorophyll *a*; TS: Total Seston; OS: Organic Seston; Phyt: Phytoplankton; STW: Somatic Tissue Weight; GTW: Gonad Tissue Weight; DGTW: Digestive Gland Tissue Weight; GI Gonadal Index; S Prot: Somatic Protein; S Lip: Somatic Lipids; S Cho: Somatic Carbohydrates; G Prot: Gonadic Protein; G Lip: Gonatic Lipids; G Cho: Gonadic Carbohydrates; DG Prot: Digestive Gland Protein; DG Lip: Digestive Gland Lipids; DG Cho: Digestive Gland Carbohydrates. Marked values are significant at $P < 0.05$

February and April 1996, and between males and indeterminate individuals as well as between females and indeterminate individuals in July 1995 and April 1996. No significant differences ($P > 0.05$) were observed in annual averages of this component between males and females, although there were significant differences between the former and the indeterminate clams. Lipids increased in winter, being very high in July 1996. Minimum values occurred in spring-summer. Indeterminate clams showed values between 6 mg and 15 mg. Significant differences ($P \leq 0.05$) between males and females were observed in September 1995 and April 1996, between males and indeterminate clams in April 1996, and between females and indeterminates in July 1995. No significant differences ($P > 0.05$) were observed when comparing annual averages between males and females, although difference was observed ($P \leq 0.01$) in comparing these with the average lipid in the indeterminate individuals. Carbohydrates (Fig. 7c) showed a maximum in females in April 1996, a value which was significantly different from those obtained for males and indeterminate clams. Both sexes showed a minimum value in January (ca. 7 mg), with indeterminate clams showing a lower concentration in winter 1995 and a maximum in autumn 1996. Significant differences ($P \leq 0.05$) existed between sexes in October 1995 and April and July 1996, between males and indeterminate individuals in July 1995, and between females and indeterminate individuals in July 1995 and April 1996. No significant differences were observed ($P > 0.05$) when comparing annual means of males and females. The only significant difference ($P \leq 0.05$) was observed when males and females were compared with indeterminate individuals. The lowest ash content occurred in December and the maximum in January. Indeterminate individuals showed values ranging from 7 to 13 mg. There were no significant differences ($P \geq 0.05$) between males and females, with significant difference ($P \leq 0.05$) only between males and indeterminate individuals in April 1996 (Fig. 7d) . There were no significant differences ($P > 0.05$) when comparing the annual averages for males and females, but there was a difference observed ($P \leq 0.05$) between the average of both males and females with that of the indeterminate clams.

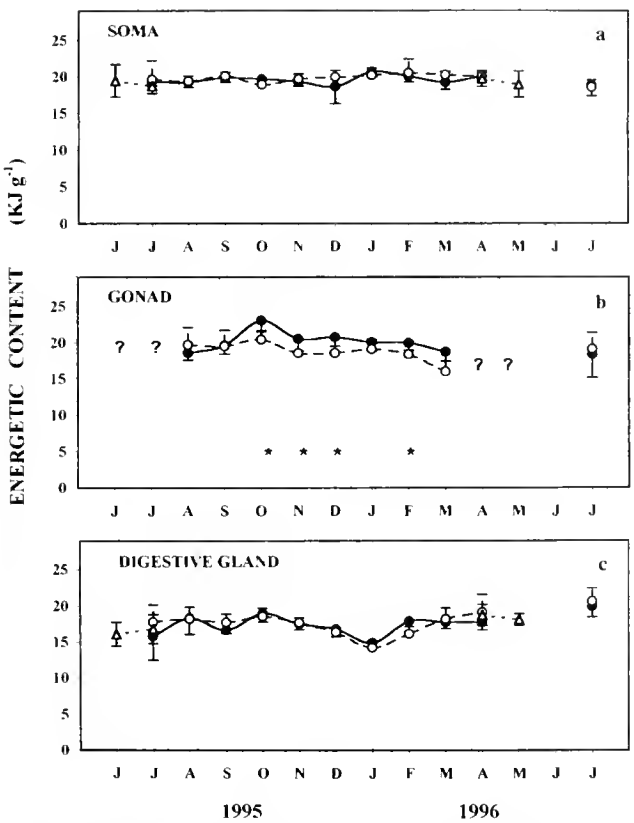

Figure 8. ***T. dombeii.*** **Temporal fluctuation in the energetic content of the different tissue. Data presented as, monthly means ± s.d. of males (●), females (○) and indeterminates (△).**
***: Significant differences between males and females at the $P \leq 0.05$ level**
?: Without information by tissue scarcity.

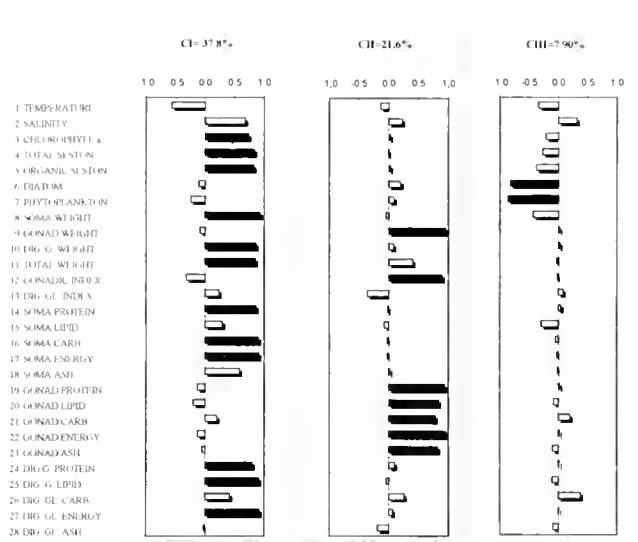

Figure 9. ***T. dombeii.*** **Principal Components analysis indicating the charge of each variable and the variance in each component. The black bars present charge ≥0.7, indicating significant association among variables.**

Energetic Content

No variation in the energetic content of the somatic tissue was observed (Fig. 8a), with no significant differences between males and females. When annual averages were compared, no significant differences ($P > 0.05$) were obtained between males, females and indeterminate clams. The energetic content of the gonad tissue increased in males, beginning in August 1995 (Fig. 8b). Significant differences ($P \leq 0.05$) were observed between males and females in October, November and December 1995 and February 1996. The annual average for males was significantly different ($P \leq 0.05$) than the average obtained for females. The energetic content of the digestive gland increased notably in July 1996 (Fig. 8c), with the minimum value for both sexes in January of this year. No significant differences ($P > 0.05$) were observed between males, females and indeterminate clams. Annual averages for energy content in the digestive gland did not show significant differences ($P > 0.05$) between males, females and indeterminate individuals.

Principal Components Analysis

The principal components analysis included 28 variables (Fig. 9), with 140 values estimated for 70 males and 70 females. Sex was not identified as an influencing factor and thus results from males and females were combined in the analysis. The variables with a charge ≥0.7 are associated with the variable component,

and as well, associated among themselves (Fig. 9). The three components explain 67.3% of the total variance. The first component associate positively chlorophyll *a*, total seston, organic seston, body weight, digestive gland weight, total weight, protein, carbohydrates and body energy content and protein, lipids, and energetic content of the digestive gland. In the second component are positively associated gonad weight, gonad index, protein, lipids, carbohydrates, energetic content and gonad ash. In the third component are positively associated diatoms and phytoplankton.

DISCUSSION

Environmental Variables

The annual cycles of temperature and salinity measured were similar to those previously described for the south of Chile (Navarro et al. 1993, Navarro & Jaramillo 1994, Stead et al. 1997, Toro et al. 1999). The concentrations of total and organic seston were lower than those reported by Navarro et al. (1993) for the tidal flat at Yaldad, (Chiloe). The total seston reached 12 $mg \cdot l^{-1}$ and the organic seston 5.6 $mg \cdot l^{-1}$, while at Yaldad, total seston reached 48 $mg \cdot l^{-1}$ and organic seston 18 $mg \cdot l^{-1}$. These differences were due to variations in primary productivity and terrigenous imports, as well as characteristics of sediments which facilitate resuspension. The highest concentrations of total and organic seston measured at Coihuin in winter 1995 were related to resuspension phenomena, in contrast with autumn-winter 1996 where they were related to the high primary production. Chlorophyll *a* rises in spring, as described for southern Chile (Navarro et al. 1993, Navarro & Jaramillo 1994, Stead et al. 1997, Toro et al. 1999) and for similar latitudes in the northern hemisphere (MacDonald & Thompson, 1985a; Navarro & Thompson Jr., 1995). A major rise was observed from April to July 1996, related with a diatom bloom. Phaeopigments showed a similar pattern to chlorophyll *a* but with lower values. In February and April 1996 high concentrations of phaeopigments were observed, probably attributable to excretion products due to an increase in grazing activities of zooplankton or, as a result of senescence of the phytoplankton (Hallegraeff 1981, Welschmeyer & Lorenzen 1985).

The phytoplankton consisted primarily of diatoms and microflagellates. The diatoms, primarily represented by *Skeletonema costatum*, showed major abundance in spring and fall, which was reflected by the increase in chlorophyll *a* as well as for total and organic seston. The greatest abundance of microflagellates occurred in December 1995 however, this was not reflected by chlorophyll *a* nor by the seston values, given the low biomass of this group.

Fluctuation in Weight and Energy Reserves

Numerous studies have demonstrated that the variation in weight in bivalves is related to reproductive cycles and environmental conditions (Thompson 1977, Sundet & Valh 1981, Jaramiilo & Navarro 1995). Temperature is commonly mentioned as a factor which influences growth and accumulation of metabolic reserves in bivalves (Bayne & Worral 1980, Vahl 1980, MacDonald & Thompson 1985a). However, Shafee (1981) concluded that the increase in weight of tissues was directly linked to the abundance of food available, Sastry 1966 and Sastry and Blake (1971), showed that temperature affected the initiation of gametogenesis in *Acquipecten irradians*, regulating the transfer of nutritive reserves from other tissues into the gonad. In *Mytilus edulis* (Thompson & Bayne 1974), *Donax trunculus* (Ansell et al. 1980) and *Modiolus modiolus* (Navarro 1990), the body weight demonstrated marked seasonal cycles with minimum values in winter and maximum in spring-summer in periods of high food availability. Conversely, *Tagelus dombeii* did not show a clear seasonal pattern, only presenting a rise in tissue weight in winter 1996, which may have been related to the unusually high concentration of diatoms occurring since autumn of that year. The situation was reflected by increase in weight of all body tissues, rise in carbohydrates and protein in somatic tissues, and in all biochemical components of the digestive gland. Interannual differences in tissue weights and cycles of metabolic reserves have been described for other species (Newell et al. 1982, Navarro 1990), and may be the result of yearly changes in environmental conditions.

The decrease in gonad weight and all their biochemical components from October to December, indicates the occurrence of a massive spawning during this period, as it was reported previously for this population by Clasing et al. (1998). The lack of increased body weight during these months, in spite of the abundance of phytoplankton, may be interpreted as a reduction in filtering activity during spawning periods as an adaptive mechanism for avoidance of ingestion of their own gametes (Newell & Thompson 1984, Thompson 1984). Also, during this period the natural rise in temperature may have resulted in higher metabolic demands.

Based on these results, the reproductive cycle of *T. dombeii* did not have a major influence on the body weight of this species. However, the initiation of gonadal development in winter 1995 coincided with a decline in the weight of the somatic tissue, suggesting that the initiation of gametogenesis occurred at the expense of reserves in this tissue. This is supported by a significant negative correlation between gonad weight and somatic carbohydrate ($r = -0.59$). Even when they were not significant at $P > 0.05$, (Table 1) a negative correlation was also observed between the gonad weight and the somatic protein ($r = -0.26$) and the lipid content of the digestive gland ($r = -0.20$). The digestive gland of *T. dombeii* did not show a decline during this season, although its lipid content decreased. Both conditions have been described for *Chlamys operculatis* (Taylor & Venn 1979) and *Placopecten maximus* (Faveris 1987) where the gonadal development in winter caused a decline in carbohydrates and protein in the adductor muscle, and a decline in lipids in the digestive gland. Under conditions of food scarcity, it has been suggested that glycogen acts as the primary energy reservoir for the formation of gametes in bivalves. Also a reduction of this reserve in storage organs is commonly correlated with an increase in gonadal lipids (Barber & Blake 1981, Benninger & Lucas 1984).

The considerable increase for gonad weight, gonadal index, and all biochemical components of the gonad of *T. dombeii* in February 1996 was related to the absorption of nutrients from the food, with significant positive correlations with chlorophyll *a*, organic seston and phytoplankton (Table 1). This suggests that this clam is able to take advantage of particulate food matter resuspended by tidal movement, waves, and winds. Minor water movement is sufficient to resuspend food particles at the bottom/water interface where this species remains buried with its siphons open at the sediment surface. Clasing et al. (1998) suggest there may be high concentrations of food material for this species at the sediment surface, given that the amount of chlorophyll *a* on the surface of the Coihuin tidal flat is somewhat greater than that encountered in the water column.

The majority of studies on the biochemical composition of bivalves describe temporal variations in components of different

tissues, comparing different populations, stages of maturation, stages in the life cycle, size classes, etc. Some studies have compared biochemical composition between males and females (Shafee 1981, Davis & Wilson 1983, Ruiz et al. 1992, Thompson & MacDonald 1990). Shafee 1981 suggested that during gonad development of *Chlamys varia*, protein was more abundant in males than in females, while these contained more lipids given the different composition of the gametes. Davis and Wilson (1983) described different levels in lipids between males and females of *Nucula turgida* only during the spawning season, with females having higher levels than males. Thompson and MacDonald (1990) found that lipids of *Placopecten magellanicus* varied from 3% to 19% in females in the spring, with no variation in males. Conversely, *T. dombeii* showed no significant differences in average monthly values for lipids between gonads of males and females. Content of protein in the male gonad was significantly higher than in the females. Carbohydrates in male and female gonads were significantly different only during the second period of gonad development which may be related to differential transformation of carbohydrates to lipids, as has been reported for other species (Gabbott 1975, Zandee et al. 1980).

Results obtained in April 1996 on comparative tissue weights between males and females suggested that the females were able to take better advantage of food availability than males, as during a large diatom bloom the females preferentially increased in weight of the fraction somatic and the digestive gland (thus total body weight). Also in this month, protein, lipid, and ash increased in female body tissues, as well as in organic components of the digestive gland. Thus, differences observed between sexes may occur during particular situations, rather than as part of annual cycles of storage and use of tissue reserves. During May, our sampling included only indeterminate individuals that had spawned were in regression, or which were initiating gametogenesis. A parallel histological study on *T. dombeii* (Clasing et al. 1998) concluded that during this month 57% of males and 80% of females were found in early active of gametogenesis, while 43% of the remaining males and 20% of the females were in the recovery stage. Also, 10% of the population was in an "indeterminate" stage, post regression and prior to gametogenesis, where sex determination could not be made. In May, indeterminate individuals underwent increases in somatic tissue weight and lesser increases in gonad weight in the presence of high diatom concentrations. The weight of the digestive gland of indeterminate individuals increased compared with males from the previous month. At that time, an increase in all biochemical components was observed in the somatic tissue, except for the lipids (gonad could not be sampled due to scarcity of this tissue). Also, a decrease was observed in proteins and carbohydrates of the digestive gland, with an increase in ash content. These observations suggested that nutrients coming from the phytoplankton were rapidly transferred from the digestive gland to other tissues as maintenance energy, for growth and to sustain incipient gametogenesis. This agrees with Gabbott (1976), who suggested that when sufficient food was available during gametogenesis, there was rapid transfer of assimilated food from the digestive gland to the other tissues. According to Thompson (1972), this transfer may occur within about seven days.

The energy available for the production of gametes may be affected by metabolic stress, such as produced by parasitism (Sanders 1966, Sanders & Lester 1981). Periodical sampling of the *T. dombeii* population at Coihuin revealed a high incidence of parasitism by a digenetic trematode (Familia Philophthalmidae), infestation could reach at times 100% of the adult population, but it rarely caused castration of individuals (<2.5%) (Yañez 1998).

In the principal component analysis, the first component associated positively the chlorophyll *a* and seston with the weight of somatic tissue and that of digestive gland, as well as with energetic content of these tissues. In the second component, the variables related to the gonad. This situation was expected, considering that the somatic tissue and digestive gland are organs of storage, constituting the main body of the organism, and that the gonad only develops in certain seasons of the year and depends as much on body reserves as on food availability.

The cycle of biochemical components in body tissues and digestive gland of *T. dombeii* at Coihuin is related to the production and liberation of gametes. Initiation of gametogenesis in winter takes place at the expense of stored tissue reserves, until food availability at the beginning of spring is able to continue to support this process. This situation has been described for *Chlamys operc*ularis (Taylor & Venn 1979), *Placopecten magellanicus* (Comely 1974, Faveris 1987) and *Mytilus edulis* (Zandee et al. 1980). Given that the increase in weight of the gonad coincides with a high condition of the organism, or elevated of food availability, gametogenesis does not provoke a decrease in body weight of individuals. Environmental variables such as temperature, which regulates metabolism, and food availability which provides necessary energy, play fundamental roles during the process of storage, distribution and utilization of energy reserves of *T. dombeii*. Temporal and spatial differences in these variables are reflected in the growth and gametogenesis of this species.

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ENERGETICS VARIATION OF THE STRIPED CLAM *EURHOMALEA EXALBIDA* (CHEMNITZ, 1795) IN USHUAIA BAY, BEAGLE CHANNEL (54°50′S)

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ABSTRACT The processes of energy transfer in the clam *Eurhomalea exalbida* Chemnitz, 1795 can be analyzed by measuring the seasonal variations in energy content with respect to the reproductive characteristics of the population. The energy content of three organ groups of *E. exalbida* (gonad, digestive gland and gastrointestinal tract-foot (FV); adductor muscles (M); and gills-mantle-siphons (GMS), was measured using calorimetry in monthly samples of the Ushuaia Bay population for a year. The relative condition index was also measured monthly for the three organ groups. No relationship was found between the energy content per gram (kJ/g ash free dry weight) of the organ groups FV, M and GMS and the size of the adult organisms. The mean values for the twelve month experimental period for FV were very similar for males and females with 20.55 ± 1.10 kJ/g for males and 20.26 ± 0.91 kJ/g for females; for M they were 20.68 ± 0.65 kJ/g for males and 20.76 ± 0.76 kJ/g for females; and for GMS they were less than for the other two organ groups, with values of 20.02 ± 1.09 kJ/g for males and 20.02 ± 0.84 kJ/g for females. An analysis of the energy content data and the relative condition index (RCI) measured at monthly intervals throughout the year revealed significantly lower values for FV in November ($p < 0.05$), which is believed to be linked to the greater gametic emission in this month. In contrast, no significant differences over time were found in the energy content or relative condition index of either M or GMS. The relative stability of the energy content and RCI of M and GMS over time suggests that there were no changes in the proportion of their biochemical components and no changes in the mass of the organs, hence *Eurhomalea exalbida* does not have an energy reservoir and the energy used in reproduction may be extracted directly from the food consumed.

KEY WORDS: energetics, clams, calorimetry, *Eurhomalea exalbida*, bivalve, Beagle Channel

INTRODUCTION

An understanding of the energy balance of an organism is based on knowledge of the temporal variation in energy acquisition and its use in maintenance, growth and reproduction. Physiological processes in temperate and cold water bivalves are also strongly influenced by seasonal variations in temperature and the availability of food (Mann 1979, Newell & Branch 1980, Smaal et al. 1997), thus patterns of growth and reproduction vary seasonally for each species. Variation in these activities has been shown to be correlated with changes in the energy content of different organs (Jobling 1994, Lucas 1996), thereby reflecting the spatial and temporal energy distribution within the organism. This distribution closely reflects the seasonality of the cost of reproduction and the capacity to accumulate reserves (Zandee et al. 1980, Robinson et al. 1981, Navarro & Torrijos 1995, Martínez & Mettifogo 1998).

Many investigations have examined variations in the energy content of different bivalve organs in relation to reproductive cycles, growth and basal metabolism. In such studies, the biochemical analysis of tissues (Beukema & De Bruin 1977, Beukema & De Bruin 1979, Zandee et al. 1980, Robinson et al. 1981, Sundet & Vahl 1981, Hérald & Deslous-Paoli 1983, Martínez & Mettifogo 1998), methods involving measures of the metabolic rates of live organisms (Newell & Branch 1980, MacDonald & Thompson 1986, MacDonald & Bourne 1987, Widdows & Johnson 1988, Sukhotin 1992, Smaal et al. 1997) and direct measures of caloric content by complete combustion (Beukema & De Bruin 1979, Griffiths & King 1979, Hérald & Deslous-Paoli 1983) have been used. Bomb calorimetry is an accurate method for measuring the energy content in aquatic organisms (Beukema & De Bruin 1979, Hartman & Brandt 1995, Lucas 1996).

Eurhomalea exalbida (Chemnitz 1795) is a species with a wide geographic distribution, from the Beagle Channel (54°50′S) to the island of Chiloe (42°S) in the Pacific littoral zone (Soot-Ryen 1959, Dell 1964, Osorio et al. 1979) and to the province of Buenos Aires (36° S) in the Atlantic littoral zone (Carcelles 1944, Carcelles 1950). *E. exalbida* is commercially exploited in various regions, although management practices are not generally used as very little is known of the clam's biology and the environmental factors that influence its populations. The Beagle Channel population represents the extreme south of the species' distribution, withstanding large temperature variations (4°C–11°C), with marked seasonal variation in the biomass of phytoplankton (Hernando, pers. comm.). These conditions may cause temporal fluctuations in the energy content of this population.

The purpose of this study was to analyze the temporal variation in the energy content of different organs in *E. exalbida* in relation to its reproductive cycle.

METHODS

Samples were collected in Ushuaia Bay (54°50′S, Beagle Channel), in a subtidal flat with a depth between two and four meters at low tide. Monthly sampling was conducted by SCUBA diving from October 1998 to September 1999. Monthly mean surface temperature was recorded. Clams with shell lengths greater than 38 mm ($n = 246$) were caught and kept in aquaria for 24 hours. Shell length (anterior-posterior axis, SL), measured with electronic calipers to the nearest 0.01 mm, and total weight (TW, ±0.1g) were recorded for each individual.

Calorimetric samples were identified and stored at −20°C until processing. After removing the valves, the sex of each individual was determined using gonad smears and the soft parts were separated into 3 groups: foot-visceral mass (gonad, digestive gland and gastrointestinal tract) (FV), adductor muscles (M), and gills-mantle-siphons (GMS). These divisions were made based on the presence or absence of gonads in the tissues and on their potential capacity to fulfill a storage function: FV includes all the tissues where gonads are present; M plays an important role as a reserve

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organ in some bivalve species; and GMS is made up of all the other tissues where gonads are not present. Although it was not possible to separate the gonad tissue from the visceral mass, the calorimetric analysis of the soft parts separated as described above was considered to give more information about the energy balance of the species than an analysis of the organism as a whole. The soft parts were dried at 80 C to constant weight.

The relationships between shell length and FV dry mass, M dry mass and GMS free dry mass are represented by 1) log (Mass) = a + b × (log (SL)). Monthly condition values were analyzed using 2) condition index (CI) = Mass/Shell length ^{b}, where b is the slope in (1), and were normalized using 3) (RCI) = (CI − mean CI)/(S.D. of CI) to give a relative condition index.

The caloric contents of approximately 20 individuals per month were obtained by burning pellets (25–200 mg) in a Parr model 1425 micro-bomb calorimeter, as described by Beukema and De Bruin (1979) and Lucas (1996). The values obtained were corrected for ash and acid contents and were expressed as kJ/g ash free dry weight (AFDW).

Statistical Analysis

A regression analysis was performed with the energy content of the different organ groups as the dependent variable and the size of the organisms (SL) as the independent variable.

The monthly differences in relative condition index and energy content in each of the groups of organs (FV, M and GMS) were analyzed using an analysis of variance (ANOVA). The assumptions of normality and homogeneity of variances were tested and the appropriate transformations were applied when necessary. When this was not possible, a non-parametric test was used (Kruskal-Wallis). Unplanned comparisons were made (STATISTICA software) when significant differences were found.

After verifying Tukey's assumption of non-additivity (BIOM-STAT software), an analysis of variance (ANOVA) for randomized block design (RBD) was used to determine if there were differences in energy content (kJ/g AFDW) among FV, M and GMS for each month and both sexes. When significant differences were found among organ groups, unplanned comparisons were made (Zar 1984, Sokal & Rohlf 1995).

RESULTS

An analysis of the relative condition index for FV over time showed the highest values in January and February in both sexes, whereas the lowest values occurred in November and April for females and November and August for males. These results were highly significant (one-way ANOVA; $F_{\alpha=0.05,\ 11,\ 109} = 2.84$ $p < 0.002$ females and $F_{\alpha=0.05,\ 11,\ 113} = 3.96$ $p < 0.001$ males; modified Tukey test, $p < 0.05$). Theses results coincide with the gametogenic cycle observed for this species (Morriconi et al. unpubl.). No significant changes in the relative condition index over the experimental period were found for either M or GMS in either sex (one-way ANOVA, $p > 0.05$, Fig. 1). The minimum mean seawater temperature (4.5 C) was recorded in August and the maximum (8.7 C) was recorded in January (Fig. 2).

Linear regressions were used to determine the possible relation between the energy content (kJ/g AFDW) of the three organ groups (FV, M and GMS) and the size of the clams (SL). The results were not significant ($p > 0.05$) for FV ($R^2 = 0.001$) and GMS ($R^2 = 0.0002$) and were significant for M although only 6% of the total variation is explained by the regression ($R^2 = 0.06$).

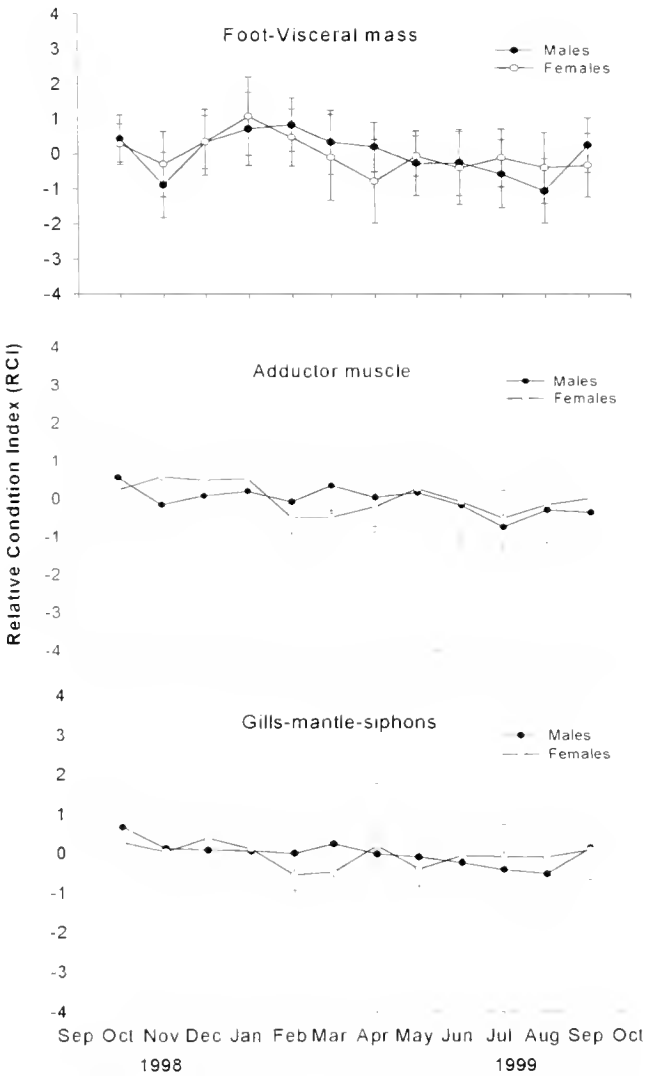

Figure 1. Mean and standard error of the relative condition index for foot-visceral mass, adductor muscles and gills-mantle-siphons over the experimental period for females and males of *E. exalbida*.

As caloric content per gram was found not to be related to the size of the organisms analyzed in this study, an analysis of variance (ANOVA) was used to analyze the variations in energy content.

Foot-Visceral Mass (gonad, digestive gland and gastrointestinal tract)

The mean energy content of FV over the experimental period for females (n = 121) and males (n = 125) were very similar (Figs. 3a and 3b respectively), at 20.26 ± 0.91 kJ/g AFDW for females and 20.55 ± 1.10 kJ/g AFDW for males.

The differences among the months were highly significant (one-way ANOVA) in both males ($F_{\alpha=0.05,\ 11,\ 113} = 4.32$, $p < 0.001$) and females ($F_{\alpha=0.05,\ 11,\ 109} = 3.39$, $p < 0.001$). It was shown in the unplanned comparisons (modified Tukey test) that the energy content for females was significantly lower in November and March ($p < 0.05$), and in October and February (the months prior to the decrease) the maximum energy content was observed although statistical significance was not obtained. The only significant variation in energy content among males occurred in November and the energy content was significantly less ($p <$

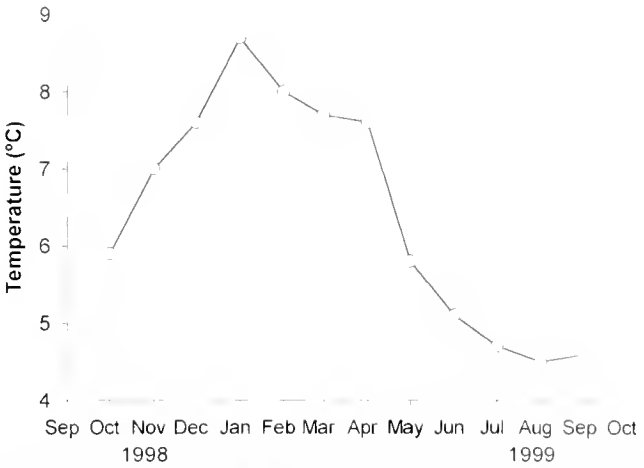

Figure 2. Monthly means of sea water temperature in Ushuaia Bay, Beagle Channel.

0.05) than in the rest of the year. Another decrease occurred in March, and in the months before these declines the greatest values were obtained.

Adductor Muscle

The mean energy content of M over the experimental period (Fig. 4a, Fig. 4b) was 20.76 ± 0.76 kJ/g AFDW (n = 121) for females and 20.68 ± 0.65 kJ/g AFDW (n = 125) for males. No significant differences were found in the energy content of M attributable to either sex ($F_{\alpha=0.05,\ 1,\ 222} = 0.08$, $p > 0.05$), time ($F_{\alpha=0.05,\ 11,\ 222} = 0.77$, $p > 0.05$), or their interaction ($F_{\alpha=0.05,\ 11,\ 222} = 0.47$, $p > 0.05$) (two-way ANOVA).

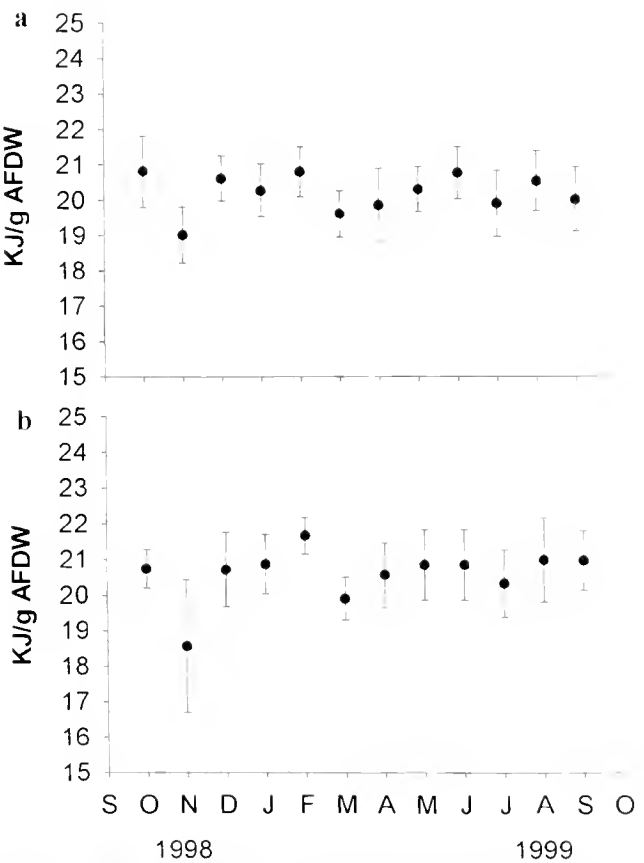

Figure 3. Mean and standard error of the energy content for foot-visceral mass (kJ/g AFDW) over the experimental period for (a) females and (b) males of *E. exalbida*.

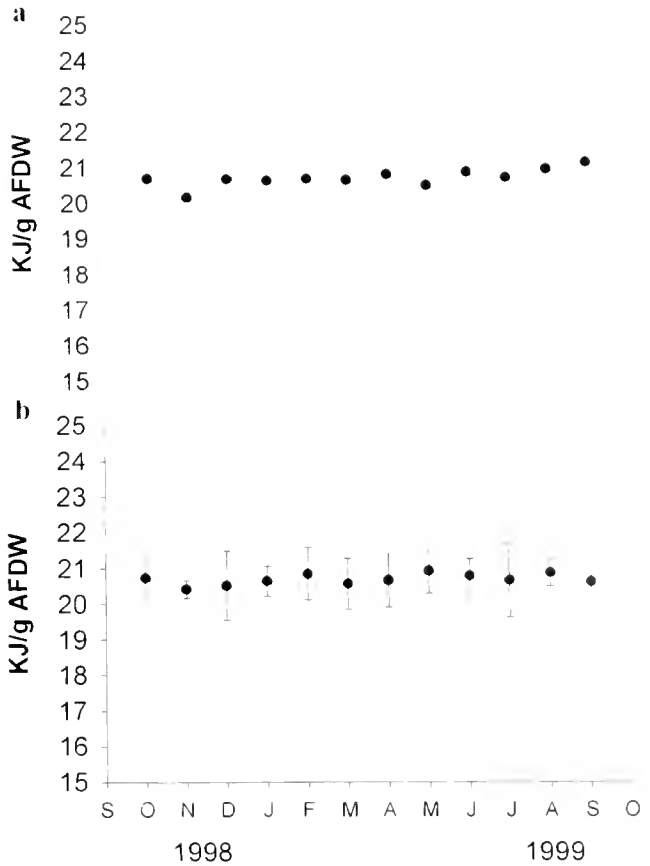

Figure 4. Mean and standard error of the energy content for adductor muscles (kJ/g AFDW) over the experimental period for (a) females and (b) males of *E. exalbida*.

Gills, Mantle, Siphons

The mean energy content of GMS over the experimental period (Fig. 5) was 20.02 ± 0.84 kJ/g AFDW for females (n = 121) and 20.02 ± 1.09 kJ/g AFDW for males (n = 125). No significant difference was found in the GMS energy content for males over time (Kruskal-Wallis; H = 16.29, $p = 0.1307$). In contrast, for females there were significant differences in energy content (one-way ANOVA, $F_{\alpha=0.05,\ 11,\ 109} = 2.58$, $p < 0.01$), with the lowest values in December and April and the maximum value in June (modified Tukey test: $p \cong 0.05$).

Variation Among Organ Groups

The monthly measures of energy content in the different groups of organs in both sexes (Fig. 3, Fig. 4, Fig. 5) show a marked stability in energy content for M, while for FV and GMS the variation was greater. A one-way ANOVA for RBD revealed significant differences ($p < 0.05$) among the energy content of FV, M and GMS (Table 1). This result suggests a difference in the biochemical composition of the organ groups.

In order to determine the organs responsible for these differ-

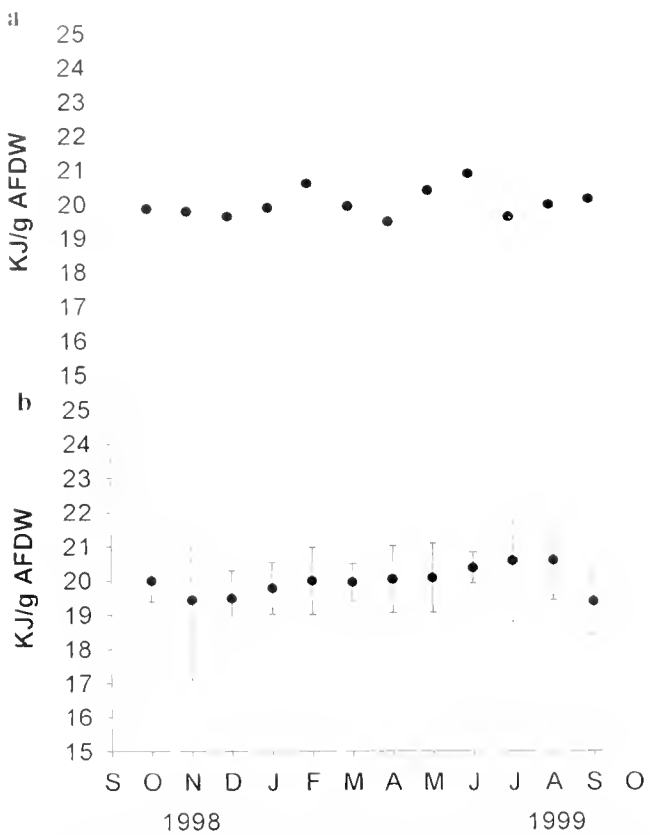

Figure 5. Mean and standard error of the energy content for gills-mantle-siphons (kJ/g AFDW) over the experimental period for (a) females and (b) males of *E. exalbida*.

ences, unplanned comparisons were made using the Tukey test. The energy content for both sexes was lower for GMS than for FV and M for most of the year, and in many instances these differences achieved statistical significance (Table 2; $p < 0.05$). The greatest difference in energy content occurred in November and March and is attributable to the large decrease in FV energy content, with lower values than for M and GMS.

TABLE 1.

Results of ANOVA (RBD) on the differences in energy content (kJ/g AFDW) among foot-visceral mass (FV), adductor muscle (M) and gills-mantle-siphons (GMS) of *E. exalbida* in Ushuaia Bay.

	Males		Females	
Date	F	p	F	p
October 1998	9.14	0.0012*	3.42	0.0553
November 1998	2.62	0.1133	4.56	0.0476*
December 1998	4.47	0.0248*	11.57	0.0003*
January 1999	8.02	0.0027*	4.17	0.0307*
February 1999	3.38	0.1382	0.31	0.7382
March 1999	5.09	0.0135*	7.46	0.0062*
April 1999	3.54	0.0424*	3.13	0.0804
May 1999	5.81	0.0088*	0.21	0.8129
June 1999	2.16	0.1377	0.15	0.8603
July 1999	0.39	0.6817	4.55	0.0235*
August 1999	0.41	0.6698	5.25	0.0136*
September 1999	10.38	0.0059*	15.23	0.0000*

F = calculated F statistic; p = probability level and (*) significant difference with $\alpha = 0.05$.

DISCUSSION

Calorimetric analysis of the energy content of different organs allows the processes of energy transfer at the individual and population levels to be understood for a given species, yet such studies in clams are scarce. In *E. exalbida*, as in clams in general, it was not possible to separate each organ, and the organ groupings used here may mask some differences among the individual organs. Notwithstanding, the results obtained have yielded valuable information about annual energy variations in specific locations within the clam.

In this study of *E. exalbida* no significant regression was found between energy content per gram and the size of the individuals being studied for FV and GMS, and for M the regression was significant although only 6% of the variation in energy content was explained by size. As the amount of energy used for growth and reproduction is different for juveniles and adults (Griffiths & King 1979, Héral & Deslous-Paoli 1983, Smaal et al. 1997), only adult individuals larger than the size at first sexual maturity (SL = 40 mm) (Morriconi et al. unpubl.) were used. Thus, the energy available for different metabolic processes per gram of tissue (AFDW) in sexually mature individuals in this study was independent of their sizes.

The energy content measured (Figs. 3, 4 and 5) in this study is consistent with the range indicated in the literature (Brey et al. 1988).

In individuals of both sexes, the gonads contained mature gametes throughout the year, indicating partial spawning (Lomovasky et al. 2000). Coinciding with a decrease in gametic abundance in November (Lomovasky et al. 2000), declines in the relative condition index (Fig. 1) and energy content (Fig. 3) of FV were observed in both males and females. Although the decline in the energy content of FV cannot be unequivocally attributed to an increase in sexual activity, it should not be rejected as a likely cause as the proportion of lipid content in the gonads can be expected to decrease due to the emission of gametes. The increase in the relative condition index and the increase in the energy content during the summer months (December, January, February)

TABLE 2.

Tukey Test for unplanned comparisons among the energy content (kJ/g AFDW) of foot-visceral mass (FV), adductor muscle (M) and gills-mantle-siphons (GMS) for males and females of *E. exalbida* in Ushuaia Bay. Only months with significant differences are shown ($p < 0.05$).

	Males		Females	
	FV	M	FV	M
GMS	October December January May September	October January March May September	December	December January March July August September
M	March		November March September	

may be directly related to the increase in the number of mature gametes in these months (Morriconi et al. unpubl.). The higher surface temperatures in the sea and the greater availability of phytoplankton (Hernando, pers. comm.) are also present in these months suggesting that these environmental factors may be related to the maturation of gametes.

In March another drop in FV energy content was observed although it was not as pronounced as the decrease in November and was not significant for males. The March values are not related to changes in the gonads and may be related to variations in the energy content of the rest of the visceral mass or the foot.

No significant variation was found in the energy content of the adductor muscles (M) over time, suggesting that there are no changes in the proportion of the different biochemical components (proteins, lipids and carbohydrates) in this tissue. Nor were significant changes observed in the relative condition index over time, thus there is no evidence of changes in the muscular mass over time. Therefore, the adductor muscles do not fulfill the function of an energy reservoir as has been shown in pectinids (Sundet & Vahl 1981, Lucas 1996, Racotta et al. 1998).

The energy values were lower for GMS than for M and FV (except in November and March), remaining almost constant throughout the year. Similarly, no significant variation was found in the RCI for GMS over time.

Seasonal variation in temperature and the availability of food appear to be closely related to the energy available for growth and reproduction in different bivalve species, such as *Mytilus edulis* (Zandee et al. 1980, Sukhotin 1992, Smaal et al. 1997), *Aulacomya ater* (Griffiths & King 1979), *Placopecten magellanicus* (MacDonald & Thompson 1986), *Macoma balthica* (Beukema & De Bruin 1977), *Tapes philippinarum* (Mann 1979), *Cerastoderma edule* (Navarro et al. 1989, Smaal et al. 1997), *Meretrix meretrix* (Jayabal & Kalyani 1986). A similar relationship has been proposed for some Antarctic invertebrates (Clarke 1980).

In *E. exalbida* the variation detected in FV energy content and relative condition index during the experimental period is related to the reproductive status of the individuals, while the minimal variation in the energy content and relative condition index of M and GMS over the same period indicates a lack of energy transfer among organs and the absence of an energy reservoir. Thus, it follows that the energy necessary for different metabolic processes may be extracted directly from the food consumed.

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BROODSTOCK MAINTENANCE AND EARLY GONADAL MATURATION OF *PHOLAS ORIENTALIS* (BIVALVIA: PHOLADIDAE)

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ABSTRACT The angelwings, *Pholas orientalis* were maintained and conditioned in indoor tanks/basins. They showed high survival in both static system and flow-through system provided with a muddy sand substrate. Enhanced gonad development and earlier maturation were observed for angelwings fed a mixture of *Chaetoceros calcitrans* and *Tetraselmis suecica* at a rate of about 294 million cells per broodstock per day. Angelwings fed a mixture of two algal species spawned one to two months earlier than those fed one algal species. Results of the study demonstrated the hatchery potentials of angelwings.

KEY WORDS: *Pholas orientalis*, angelwings, broodstock, gonadal maturation

INTRODUCTION

Several years ago, the high demand for angelwings, *Pholas (Monothyra) orientalis* locally known as diwal in Panay and Negros Islands in Central Philippines resulted in the overexploitation of the species. The once dense beds are now almost devoid of this resource. Certain actions or interventions must be undertaken so that these areas would again become an important sustainable source of income for coastal fishermen.

The most active strategy in assisting improvement and rehabilitation of coastal mollusk fisheries is through stocking of artificially produced seedlings. This has been demonstrated in many eastern Asian maritime countries. In Japan, the production of *Haliotis* spp., *Paphia* spp., and *Pecten* sp. have increased tremendously (Honma 1980, Inoue 1984, Oshima 1984). In Taiwan, increased commercial productions were attained for shellfishes like *Meretrix lussoria*, *Gomphina veneriformes*, *Haliotis diversicolor* var. *supertexta*, *Solctellina diphos* and *Anadara granosa* (Chen & Yang 1979, Chen 1984, Ting 1984).

The Philippines lag behind in techniques of seed production and restocking of coastal areas to increase and sustain shellfish production compared to its neighboring countries. The main reason is the absence of molluscan hatchery, which is crucial and complementary to a rehabilitation program. We view that even hatchery-related studies are limited and mainly focused on oysters, mussels and scallops. Research on broodstock maintenance, spawning and nursery techniques for endemic bivalve species such as angelwings has not been given priority.

This study is the first effort to determine the hatchery potential of angelwings, *P. orientalis*. The feasibility of maintaining spawners of this species under laboratory conditions was evaluated. Further, we tested the influence of different types of algal species to bring about gonadal maturation and spawning in the laboratory.

MATERIALS AND METHOD

Spawner Maintenance

Adult angelwings (50–130 mm in shell length) were collected from the intertidal flats of Barotac Nuevo, Iloilo in Central Philippines (122°47′N and 10°55′E) that earlier was described by Laureta and Marasigan (2000). The specimens were scrubbed free of fouling organisms and debris prior to stocking.

Two holding techniques were evaluated for spawner maintenance in the laboratory: (a) static water system with aeration and without substrate sediments, and (b) flow-through water system with muddy sand substrate. All incoming water was filtered through sand. Three 40-l capacity circular white basins were used in the static system and three 30-l capacity fiberglass tanks were used in the flow-through system. Ten pieces of angelwings were placed in each container. The bivalves were laid horizontally in static systems and those in the flow-through systems were buried in the muddy sand substrate. During the two months of holding, the angelwings were fed either *Chaetoceros calcitrans*, *Tetraselmis suecica* or their combination. The algae were provided to the angelwings in the morning and afternoon at the rate of about 100 million cells per adult bivalve per feeding. The angelwings were reared in ambient light and temperature (range: 24–27°C) conditions of the laboratory with minimal disturbance. Survival of angelwings was evaluated after two months. Survival of the angelwings between the two holding techniques was compared using analysis of variance (Zar 1984).

Gonadal Development

Experimental Animals and Set-up

A total of 160 angelwings (>70 mm shell length) collected at five different dates in Barotac Nuevo were scrubbed free of fouling organisms and acclimated to feed on test phytoplankton species, *T. suecica* or *C. calcitrans*. Ten specimens were dissected and subjected to histological preparations to determine angelwings' stages of maturity and gametogenic cycles before the onset of the feeding experiment. Permanent mounts of the gonads were prepared following the modified Bell and Lightner (1989) method. The stages of gonadal development were described following stages for other clams (Rossell 1979, Jones 1981, Hesselman et al. 1989, Shafee & Daoudi 1991, Panurovsky & Yakovlev 1992) and the earlier description on reproductive stages of the angelwings by Laureta and Marasigan (2000). All ten specimens were found to be spent.

Fifteen cylindrical concrete tanks (diameter = 0.5 m; height = 0.75 m) with standpipes at the center filled with 0.3 m compact muddy sand and supplied with seawater to a maximum depth of 0.17 m by means of a flow-through water system, were used in this study. Each tank was stocked with ten angelwings by burying them in the muddy sand substrate. Water physico-chemical parameters in the culture tanks were monitored regularly. Water temperature was measured using mercury thermometer and salinity was monitored using Atago refractometer.

Experimental Treatments and Protocol

The study tested the effects of different algal food on the gonadal maturation of angelwings. The algae used were (a) *C. calcitrans*; (b) *T. succica*; and (c) combination (1:1) of the two algal species. Algae were given to the angelwings twice a day (morning and afternoon) at the rate of approximately 1.47×10^9 cells tank^{-1} feeding^{-1} or 2.94×10^9 cells tank^{-1} day^{-1}. Since 10 angelwings were stocked in each tank, each angelwing was presumed to receive 294 million cells day^{-1} during the first month. The daily provision of algal food for each tank remained the same throughout the experiment but algal food allotment for each angelwing increased every month because of decreased number of angelwings in each tank due to sacrifice sampling. Each of these feeding regimes was replicated five times and assigned at random to the 15 concrete tanks. Water flow was stopped for about two hours during feeding.

Evaluation of Gonadal Development

Gonad developments of angelwings fed algal diets were monitored every month for five months. One angelwing was extracted and dissected from each tank representing five replicate samples for each type of algal food. The histological preparation and method of determining gametogenic cycles as described earlier were used. The gonadal stages were: early active, late active, ripe, partially spent and spent. The percentage distribution of gonadal stages in each treatment diet was calculated. The gonad index was estimated by assigning rank for each stage modified from Wilson (1987) and King et al. (1989): 3 for early active, 4 for late active, 5 for ripe, 3 for partially spent, and 1 for spent. The gonad index of angelwings in each treatment diet per monthly observation is the sum of products of percentage of each stage (P_s) and its rank (R_s), giving value from 100 (all spent) to 500 (all ripe).

$$GI = P_s \times R_s$$

RESULTS

Spawner Maintenance

High survival of angelwings was obtained for broodstocks maintained in both static system with aeration (90 ± 0.0%) and the flow-through system with muddy sand substrate (93.3 ± 5.8%). There was no significant difference ($p > 0.05$) between the two systems. The spawners in both holding containers were agile and active as exhibited by constant protrusion of their siphons especially during feeding time.

Gonad Development

The percentage distribution of gonadal stages and the gonad index of angelwings after feeding different algal species in the laboratory are shown in Table 1 and Figure 1. The same table contains Laureta and Marasigan's (2000) percentage distribution of the combined gonadal stages of male and female angelwings collected at different sampling dates in their natural habitat for comparison purposes with the above. Gametogenesis started in angelwings indoor one month (Jan.) after the start of the experi-

TABLE 1.

Percentage distribution of gonadal stages of *P. orientalis* fed *Chaetoceros calcitrans* (Cc), *Tetraselmis succica* (Ts) and their combination (CT) in the laboratory, and samples from a natural population *(Barotac Nuevo, Iloilo, Central Philippines).

Date	Site	Diet	N	Gonadal Stages				
				Early Active	Late Active	Ripe	Partially Spent	Spent
Dec. '94	Indoor	Cc	5	0	0	0	0	100
		Ts	5	0	0	0	0	100
		CT	5	0	0	0	0	100
	Barotac Nuevo		15	20	47	27	6	0
Jan. '95	indoor	Cc	5	20	0	0	0	80
		Ts	5	20	0	0	0	80
		CT	5	20	0	0	0	80
	Barotac Nuevo		14	29	50	21	0	0
Feb. '95	indoor	Cc	5	60	0	0	0	40
		Ts	5	60	0	0	20	20
		CT	5	60	20	0	0	20
	Barotac Nuevo		12	16	42	42	0	0
Mar. '95	indoor	Cc	5	40	60	0	0	0
		Ts	5	40	60	0	0	0
		CT	5	0	40	60	0	0
	Barotac Nuevo		9	0	44	56	0	0
Apr. '95	indoor	Cc	5	0	80	20	0	0
		Ts	5	0	60	40	0	0
		CT	5	0	0	80	20	0
	Barotac Nuevo		6	0	33	67	0	0
May '95	indoor	Cc	5	0	20	60	20	0
		Ts	5	0	0	80	20	0
		CT	5	0	0	40	40	20
	Barotac Nuevo		16	0	25	56	19	0

Data extracted and recomputed from Laureta and Marasigan (2000).

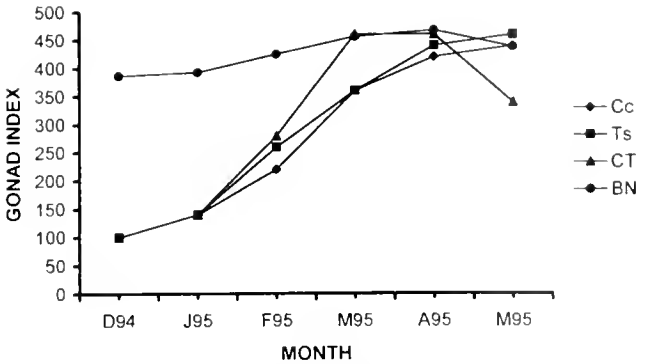

Figure 1. Gonad index of *P. orientalis* fed *Chaetoceros calcitrans* (Cc), *Tetraselmis suecica* (Ts) and their combination (CT), and samples from a natural population in Barotac Nuevo, Iloilo, Central Philippines.

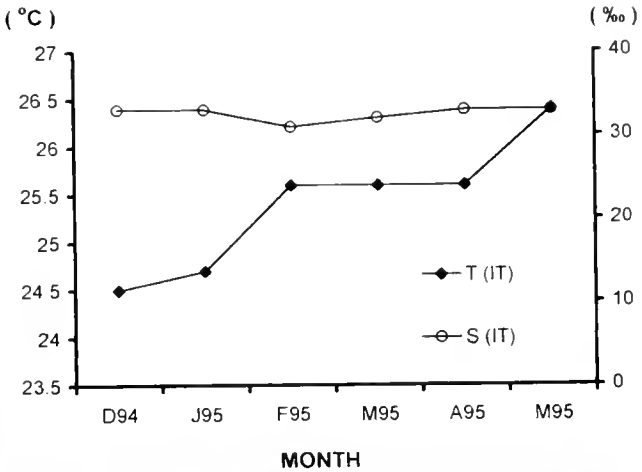

Figure 2. Variations in the temperature and salinity readings of the water in the indoor tanks from December 1994 to May 1995.

ment when 20% of the animals showed early active development. As the conditioning progressed from February through May, there was an increased percentage of mature angelwings that spawned later.

Angelwings fed combination of *T. suecica* and *C. calcitrans* showed faster gametogenesis, earlier maturation and spawning than angelwings fed one algal species. Sixty percent of the angelwings fed the mixed algal diet had ripe gonads by March while majority of angelwings fed either *C. calcitrans* or *T. suecica* did not attain the same gonadal stage until May. Angelwings fed combined algal species began spawning in April and continued in May while angelwings fed one algal species did not spawn until May. The highest histological gonad index of 460 was attained in March by those fed the combined species and in May by those fed single algal species

Angelwings in the natural habitat had advanced gonadal stages (Table 1) and higher gonad index (Fig. 1) compared to angelwings in indoor tanks at the start of the experiment. Although a similar proportion was ripe in the laboratory and the field in March, angelwings in indoor tanks spawned one month earlier (Apr.) than in natural habitat. Some of the angelwings fed combined algal species were already spent in May while in the natural population were only partially spent.

Water Physico-chemical Parameters

Water temperature in the culture tanks ranged from 24–26°C and salinity ranged from 31 to 33 (‰) (Fig. 2). No marked fluctuation was observed in the values of the parameters obtained during the culture period.

DISCUSSION

In this study, we reared and carried out gonadal maturation of angelwings in the laboratory. The similarity of survival rates between angelwings reared in circular basins without mud substrate and angelwings in tanks with mud substrate that accommodate the burrowing behavior of the species could be attributed to the minimal disturbance in both holding systems. Both systems minimized stressful conditions to the angelwings resulting in high survival and relatively faster gonadal development. Optimum holding conditions such as filtered seawater, gentle aeration, reduced water turbulence and ample supply of algal food enabled the bivalves to be active during the daytime for feeding as evidenced by continuous protrusion of their feeding siphons. In their natural habitat, the angelwings are subjected to environmental perturbations brought about by substrate instability caused by water turbulence, tidal fluctuations and fishing activities. In the field, we observed that angelwings in the coasts of Barotac Nuevo moved up and extended their siphons out of the sediment only during calm periods to feed. If dislodged by fishing i.e., trawls and dredges or turbulence due to typhoons, the angelwings could no longer get back to their burrows/homes (Ablan 1938, Laureta & Marasigan 2000). Further, we also noted that they were unable to create another burrow by themselves once exposed, thereby subjecting them either to physical stress such as water current and strong waves that usually damage and break their shells or predation.

Our results showed that angelwings could be conditioned to an earlier gametogenesis by feeding them high ration of single-cell algal diets. Earlier studies were successful in conditioning bivalves to sexual maturity through manipulation of temperature and increased food supply (Loosanoff & Davis 1963, Paon & Kenchington 1995). Apparently, the two types of algal food at a high ration fed twice a day per adult angelwing possibly satisfied the energy demanding process of gonadal maturation in shellfish (Sastry 1979). In an earlier study, Sastry (1968) reported that post-spawning adults of *Argopecten irradians* required an abundant food supply for initiation of gonadal growth and gametogenesis. The ration of 294 million cells/animal/day used in our study was higher than the highest feeding ration i.e., 67 million cells/animal/day of *Chaetoceros gracilis* and c-iso used by Villalaz (1994) to condition scallop, *Argopecten ventricosus*. The amount of algae fed to the angelwings approximated the recommended ration by Utting and Spencer (1991). However, our ration was apparently lower than the feeding ration totaling 400 million cells/day of *Thalassisiora pseudonana* fed to every broodstock of *Crassostrea gigas* by Muranaka and Lannan (1984).

The feeding mixed (1:1) algal species of *C. calcitrans* and *T. suecica* enhanced gonadal development and maturation of *P. orientalis*, and spawning which occurred one to two months earlier compared to individuals fed only one algal species diet. This may be attributed to the complementation of polyunsaturated fatty acids provided by two or more algal species as compared with a single algal species (Walne 1970, Helm 1977, Webb & Chu 1982, Gallardo et al. 1992). Robinson (1992) reported that mixing of differ-

ent algal species of unicellular algae as feed for broodstock oysters provide greater variation of fatty acid than from one species of algae.

Thus, the reduction of environmental stresses and the provision of combination of two species of single cell algae to angelwings reared indoor influenced its faster gonadal development and maturation compared to angelwings found in the natural habitat. Although we did not include the interaction of temperature and food supply, our results showed earlier maturation of laboratory reared angelwings despite the lower temperature compared to the temperature in the natural habitat (Laureta & Marasigan 2000). It appears that variation in water temperature ranging from 24 to 29°C was tolerable to the gonadal maturation of the angelwings due to high food concentration, which satisfied the angelwings requirements for development process. Earlier studies by MacDonald and Thompson (1985) also found that rapid tissue growth and shell increment of giant scallop *Placopecten magellanicus* were correlated to food availability rather than warmer temperature of the water column of Newfoundland and New Brunswick coasts. Our observations on the earlier or faster gonadal development of angelwings provided with high algal ration in the laboratory were in agreement with the observations of MacDonald and Thompson (1985) that growth of the giant scallops largely depended on reduction of nutritive stress and not necessarily influenced by warmer seawater temperature. Thus, it could be assumed that slower gonadal development and maturation of angelwings in the natural environment might be limited by the food supply.

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THE REPRODUCTIVE CYCLE OF THE NEW ZEALAND VENUS CLAM *RUDITAPES LARGILLIERTI*

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ABSTRACT The reproductive cycle of the subtidal native New Zealand venerid clam *Ruditapes largilliertı* was studied over a 16-month period in the Whangateau Harbour, northeastern New Zealand. Gametogenic development between males and females was synchronous. *Ruditapes largillierti* was found to have an annual cycle, with gametogenesis beginning in autumn (March to May), and spawning over an extended period commencing in August and ending in early summer (December). Successful fertilization of gametes was verified by observations of settlement of postmetamorphosed spat onto spat catching bags suspended in the water column in the vicinity of the Whangateau Harbour at the end of the spawning season. The study population was dominated by large adults (45–55 mm shell length). Sex ratios between males and females were equal, and there was no evidence of hermaphroditism. The reproductive cycle described here is similar to other venerid and mesodesmid clams occurring in northern New Zealand.

KEY WORDS: clam, mollusca, *Ruditapes largilliertı*, reproduction, veneridae

INTRODUCTION

Although the export of cultured bivalves (made up almost exclusively of the Pacific oyster, *Crassostrea gigas*, and the green lip mussel, *Perna canaliculus*) is worth NZ$180m annually, the potential for culturing New Zealand clams for export remains relatively unexplored. In addition, there is known to be a significant amount of recreational harvesting, the extent of which is often underestimated (Kearney 1999). Venerid clams, in particular, are likely to play an important part in the growth of future shellfish industries because of the large and well established international markets for clam species from this family. At present, the only venus clam commercially harvested in New Zealand is the cockle, *Austrovenus stutchburyi*, for which a total of approximately 100t were exported in 1999 harvesting season (NZ Seafood Industry Council 2000). It is also the most commonly harvested recreational species. However, another species of venerid clam, *Ruditapes largillierti*, may be suitable for commercial exploitation (Maguire 1991) because of its similarity to the Manila clam, *Tapes philippinarum*, the only clam for which ubiquitous global markets exist (Manzi & Castagna 1989). *Ruditapes largillierti* was collected by indigenous (Maori) harvesters.

Investigation of the seasonal reproductive cycle of any marine bivalve is essential in developing a management strategy for a fishery, whether it is commercial or recreational (Shaw 1965, Manzi et al. 1985). The success of any hatchery-based commercial venture is also ultimately dependent on knowledge of reproductive cycles and an ability to spawn broodstock (Eversole 1989, Hooker & Creese 1995).

By following the progress of gonad and gamete production, researchers can determine the timing and duration of spawning events (Ropes & Stickney 1965, Corni et al. 1985, Hooker & Creese 1995). A knowledge of spawning periods is also needed for assessing larval abundance (Ropes & Stickney 1965), predicting periods of annual recruitment (Keck et al. 1975, Manzi et al. 1985) and for interpreting growth rates and mortality data (Keck et al. 1975, Baron 1992). Ultimately, all these factors influence the distribution and abundance of juvenile and adult populations (Shaw 1965, Hooker & Creese 1995).

Histological techniques are the most commonly applied methods for determining the reproductive cycle of clams. Although they can be expensive and time consuming, histological techniques are the only reliable way of documenting gametogenic processes (e.g., Shaw 1962 and Shaw 1965, Ropes & Stickney 1965, Adachi 1979, Eversole et al. 1980, Mann 1982, Robinson & Breese 1982, Ropes et al. 1984, Manzi et al. 1985, Rosenblum & Niesen 1985, Knaub & Eversole 1988, Rowell et al. 1990, Ponurovsky & Yakovlev 1992, Hooker & Creese 1995). Histological sections can be used for both qualitative assessment and quantitative analysis of gamete development, typically using measures of oocyte size. Eversole (1989) contends that comparisons of gametogenic cycles among species would be easier if more quantitative measures of gonadal development were used (e.g., Kennedy & Battle 1964, Heffernan & Walker 1989). In the present study, both qualitative and quantitative measures were used to assess gonad development of *R. largillierti* in a northeastern New Zealand harbor.

The information presented in this paper provides the first description of the reproductive cycle of *R. largillierti* in New Zealand using both qualitative and quantitative methods, and explores whether or not the patterns observed are part of an annual cycle.

MATERIALS AND METHODS

The reproductive cycle of *R. largillierti* in the Whangateau Harbour (northeastern coast of New Zealand's North Island; Fig. 1) was documented using histological analysis of samples collected monthly from October 1996 to October 1997. An additional sample was collected in January 1998. Thirty adult clams were collected subtidally in 3–7 m of water each month using SCUBA, and transported to the Leigh Marine Laboratory, approximately 4 km from the Whangateau Harbour (Fig. 1). Clams were processed within two hours of arriving at the laboratory.

The length (anterior-posterior axis) of each clam was measured to the nearest millimeter using vernier calipers. Following this the shell was pried open using a small knife and the visceral mass removed and fixed in Bouin's solution. After allowing five days for the sample to harden the visceral mass was extracted, and the siphons, mantle and gills removed. The remaining visceral mass, with associated gonad, gut and attached foot, was then placed in a histological cassette and preserved in 70% ethanol.

Samples were dehydrated using a graded ethanol series, blocked in paraffin wax and sectioned at 7 μm. Three longitudinal

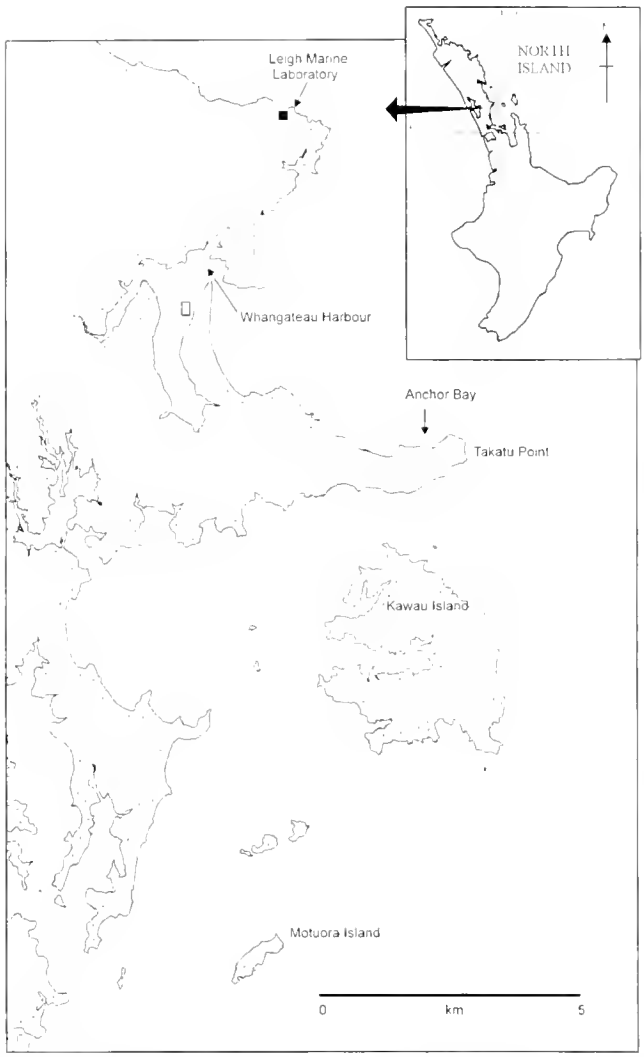

Figure 1. Map showing the location of the Whangateau Harbour, where clams were collected for reproductive analysis from October 1996 to January 1998, and the location of spat catching bags placed at Motuora Island and Anchor Bay, from August 1997 to January 1998. Scale bar = 3 km.

sections (along the anterior-posterior axis) were taken from each sample. All sections were stained using Haematoxylin and counterstained with Eosin. The histologically prepared slides were then examined using a compound microscope at ×40, ×100 and ×400 magnification. Gonads from both male and female clams were placed into five qualitative categories adapted from Porter (1964), Keck et al. (1975) and Ropes (1968): early active, late active, ripe, partially spawned and spent (Table 1; Fig. 2A–J). The gonadal state of each clam was described as one of the five stages based on the most dominant stage present in 10 randomly selected follicles from each sample.

The sex of each clam was determined from microscopic examination of the histological slides. Clams were deemed sexually mature if any mature gametes were present. A Chi-squared goodness of fit test ($\alpha = 0.05$) was used to test the hypothesis that there was an equal representation of males and females in this population. Clams were also examined for any evidence of hermaphroditism as some species of venerid clams are known to have male and female gametes co-occurring within the same individual.

Monthly mean oocyte diameters were determined using video image analysis (Mocha Image Analysis 1.2; Jandel Corp. 1994) in order to validate the gametogenic development of female clams. The diameters of all oocytes within three haphazardly selected follicles from each of the three slides were measured for 10 female clams sampled in each month. Only oocytes with visible nuclei were measured.

Interpretation of spawning events from histological analysis is based on the qualitative assessment of gonad staging. In order to validate spawning events deduced from the histological stagings, a spat catching experiment was established in Anchor Bay (174°50.5′E, 36°30′S) approximately 4 km from the Whangateau Harbour, and adjacent to Motuora Island (174°48′E, 36°30′S), approximately 15 km from the Whangateau Harbour (Fig. 1). Anchor Bay was chosen as water exiting the Whangateau Harbour circulates through this area (Parr 1993). Motuora Island was chosen as it is known to be a sink for bivalve larvae (Morrison 1998). It was not possible to put spat catching bags in the Whangateau Harbour because of the strong tidal currents and the high recreational use of the harbor.

Three 25 m drop lines were anchored to the seafloor in each region. Three 0.5 m^2 replicate mesh bags were placed at intervals of 5 m, 10 m and 15 m above the seafloor on each drop line by SCUBA in August 1997. The meshbags were collected and replaced in September 1997 and October 1997. The final set of bags was removed from the lines in January 1998 and not replaced. All bags were transported back to the laboratory and individually searched for the presence of newly settled clams.

RESULTS

Reproductive Cycle

Despite slight differences in gamete development between males and females, the reproductive development of both male and female clams followed similar patterns (Fig. 3a, b) which appeared to be related to changes in sea-surface temperatures (Fig. 3c). During 1997, gametogenic development began in March/April (early autumn): a period of decreasing water temperatures. Gametes continued to develop through August and September (winter) with ripe individuals first observed in the coldest winter months. Spawning began in August with a small number of both male (20%) and female (7%) gonads appearing partially spent. Spawning activity increased as sea-surface temperatures continued to rise. By October 1997, 70% of males and 82% of females appeared in a partially spent or spent condition. This pattern was also observed a year earlier in October 1996, although the spawning event seems to be stronger with all clams in either a spent or partially spent condition (Fig. 3a, b). During the 1996/1997 spawning period, all clams were completely spent by December 1996. Although clams were not sampled in December 1997, this pattern appears to be repeated during 1997/1998 with all clams having spent gonads by January 1998.

Oocyte Development

The mean monthly diameter of oocytes ranged from 21.3 μm (February 1997) to 31.9 μm (March 1997) (Fig. 4). The peak value in March 1997 was due to the small number of residual oocytes that hadn't yet been resorbed. From October 1996 to January 1998 mean monthly diameters alternated regularly between high and low values. Although there does not appear to be a strong rela-

TABLE 1.

Criteria used to stage histologically prepared slides. Adapted from Porter (1964), Keck et al. (1974) and Ropes (1968).

Stage	Males	Females
Early active	Follicle walls thick, lined with a thick layer of spermatogonia occupying up to a third of follicle area. Spermatocytes and spermatids develop in the middle of the follicle. Spermatozoa, with tails pointing into the lumen, often occupy the center of the follicle. Gonad volume is small.	Follicle walls thick, often contracted. A lot of ovogenic activity with many oogonia and primary oocytes attached to the follicle walls. Mature oocytes and ova present. Gonad volume is small. There is often a lot of connective tissue visible within the gonad.
Late active	Follicle walls are thin. Spermatogonia restricted to lining the follicle walls. Follicle dominated by dense areas of spermatids and spermatocytes. Spermatozoa more abundant than early active.	Follicle walls are not as thick. There are a lot more ova and mature oocytes than in the early active stage, often attached to the follicle wall by a thin stalk. There are fewer primary oocytes. Mature oocytes are often rectangular or polygonal in shape.
Ripe	Spermatogonia as for late active. Follicle dominated by very dense spermatozoa with tails pointing into the lumen. Spermatids and spermatozoa occupy less follicle volume. Gametes occupy nearly all the gonad volume.	Follicle walls are thin and full of ova often lying free in the lumen. There is little ovogenic activity within the follicle except for a few mature oocytes. Ova are usually spherical in shape.
Partially spawned	Spermatozoa still occur in the follicle but with large gaps. Center of the lumen often appears empty. Spermatogonia intrude further into the follicle although no more abundant than in the previous stage. Spermatids and spermatocytes less dense but still fairly common.	Follicles are large with walls that are very thin and often ruptured. There are usually large spaces within the lumen, although free ova are still frequent within lumen of the follicle. There are still mature oocytes present.
Spent	Follicle walls are thickened. Few spermatogonia still present, with few unspawned spermatids, spermatocytes and spermatozoa still remaining. Gametes occupy very little of the gonad volume.	Spawned: The follicle is essentially empty with a few ova still free in the lumen. Follicle walls may be contracted and thickened with connective tissue.

tionship between stage of reproductive development and mean monthly oocyte diameter, peak mean oocyte values occurred during June 1997, a month dominated by developing late active oocytes, and September 1997, which is dominated by ripe and late active oocytes.

Analysis of the monthly frequency histograms for oocyte diameters indicates egg size ranged from 10 to 27 μm and that a broad range of egg sizes was present in all months sampled (Fig. 5). Again, there does not appear to be a strong association between the relative frequencies of egg diameters in each mode and stage of reproductive development.

Sex-Ratio

Adults collected during monthly sampling ranged in size from 29–58 mm shell length, and all could be sexed. The Chi-squared test performed on the monthly and total number of males and females sampled failed to detect any significant difference from a ratio of 1:1 (Table 2). However, the sex ratio of the 10 clams collected ≤35 mm (3 males and 7 females) was 1:2.33.

Size at Sexual Maturity

Length (longest anterior-posterior distance) was used to assess size-at-sexual maturity. All clams collected were mature. Males and females had almost identical size ranges and mean shell lengths in all months sampled (Table 3). Males ranged in size from 29–57 mm and females ranged from 29–58 mm (Table 3). The mean monthly length of males ranged from 44.3 (±7.51) to 51.3 (±1.5) and from 44.0 (±9.4) to 51.1 (±2.9) for females.

Occurrence of Newly Metamorphosed Juveniles

Twelve *R. largillierti* spat were recovered from the series of meshbags retrieved from Anchor Bay in January 1998. These ranged in size from 0.5–1.5 mm in length. The presence of *R. largillierti* spat at this time correlates well with the spawning period evident from the histological stagings in the 4 months prior to January 1998. However, no post-metamorphic clams were found on any of the bags recovered from Motuora Island.

DISCUSSION

Temperature is often regarded as the main factor determining the general patterns of reproductive development and spawning. Indeed, many species experience latitudinal gradients in the timing of reproductive development and spawning events, and also in the number of spawning events associated with changes of temperature (e.g., Keck et al. 1975, Eversole 1989, Heffernan et al. 1989). As with other species of clams, the reproductive cycle of *R. largillierti* in the Whangateau Harbour appears correlated to changes in sea-surface temperature. Gamete development began in autumn (March–April) as seawater temperatures started falling, with ripe individuals first present when temperatures were at their lowest during July. Spawning commenced in August as seawater temperatures began rising, although the majority of spawning appeared to occur during the months of October and November. Paturusi (1994) found a similar pattern of reproductive development for *R. largillierti* in Georges Bay, Tasmania. The native geoduck, *Panopea zelandica*, collected from the Coromandel Peninsula from June 1999 to March 2001 also went through a similar reproductive cycle to that of *R. largillierti*, with spawning beginning in early spring and ending in early summer (Gribben unpub. data). Spawning of the pipi, *Paphies australis* (Hooker & Creese 1995), and the tuatua, *P. subtriangulata* (Grant & Creese 1995), in the Whangateau Harbour also began in early spring, however, it continued right through summer.

Although the reproductive cycle was correlated to sea-surface temperatures, low temperatures during the winter, particularly in

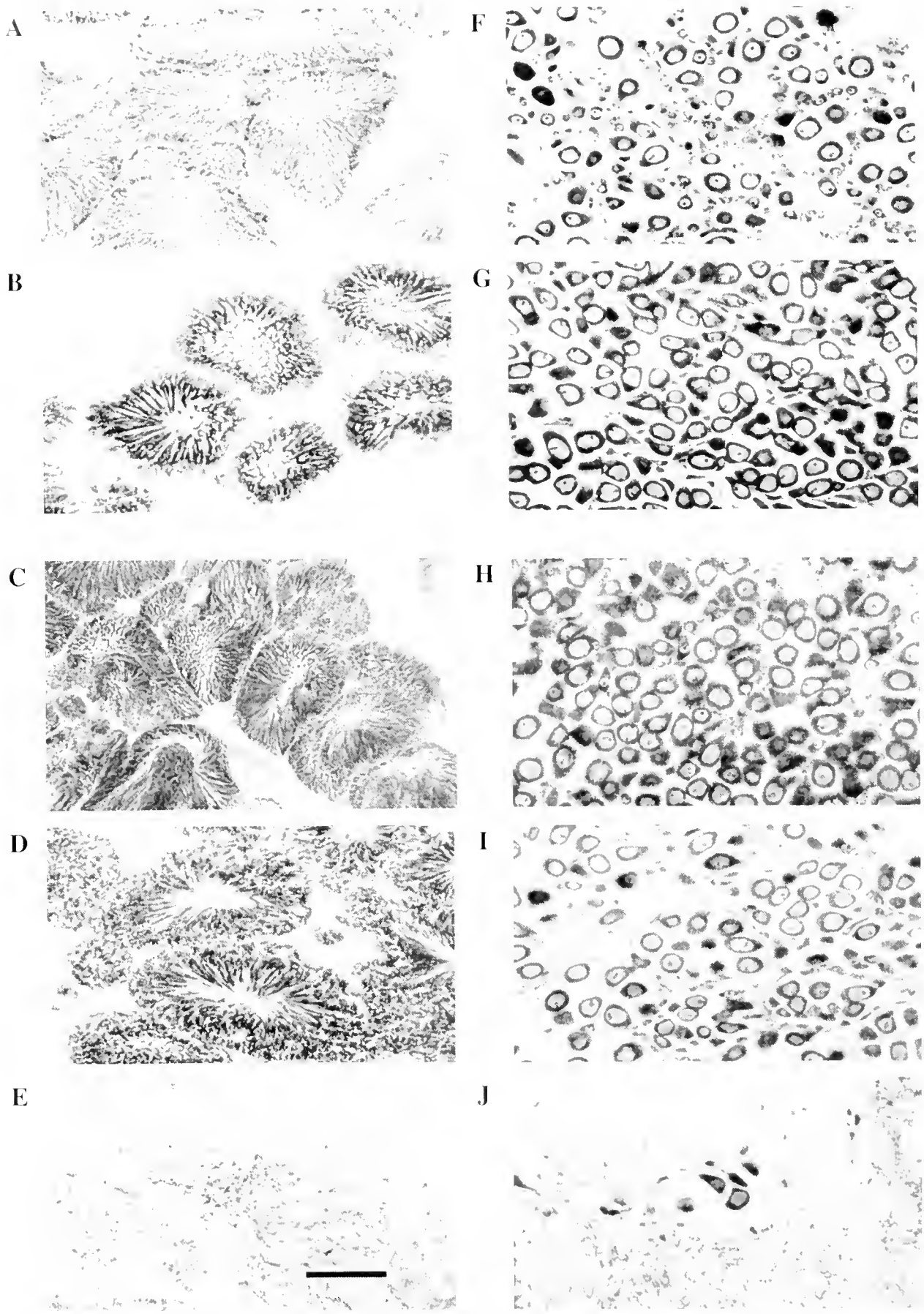

Figure 2. Photomicrographs of reproductive stages; Males (A) early active, (B) late active, (C) ripe, (D) partially spawned, (E) spent; and females (F) early active, (G) late active, (H) ripe, (I) partially spawned, (J) spent. Scale bar on E = 100 μm, and applies to all micrographs.

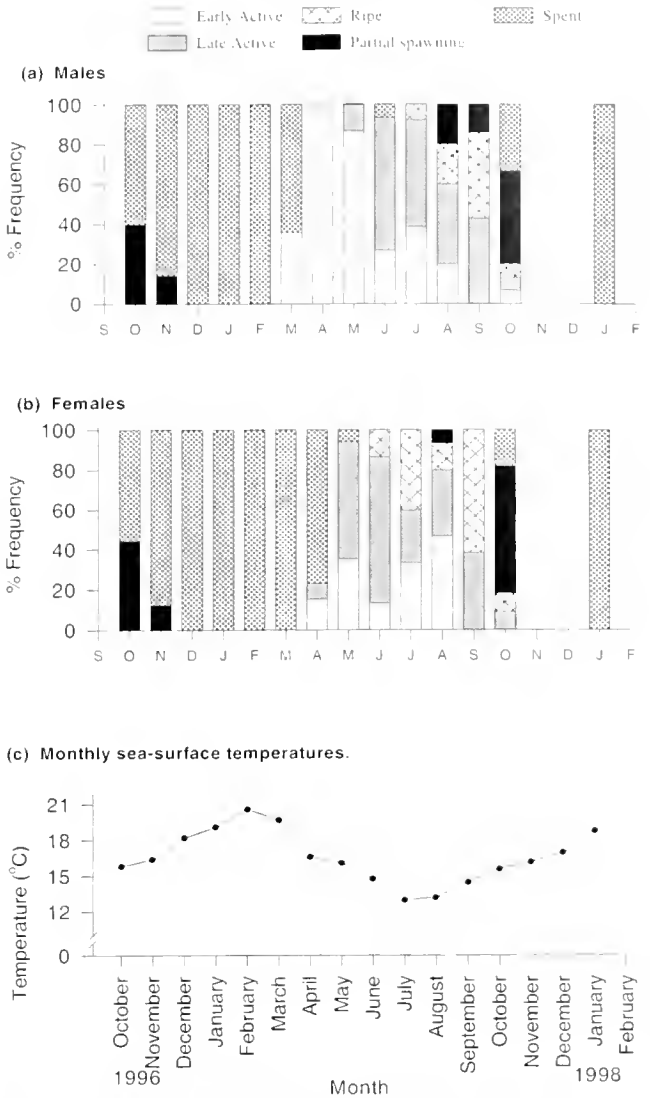

Figure 3. Histograms showing the gametogenic cycle of *R. largillierti* for males (a) and females (b) determined from analysis of histological sections. (c) Monthly temperatures recorded at the Auckland University Marine Laboratory.

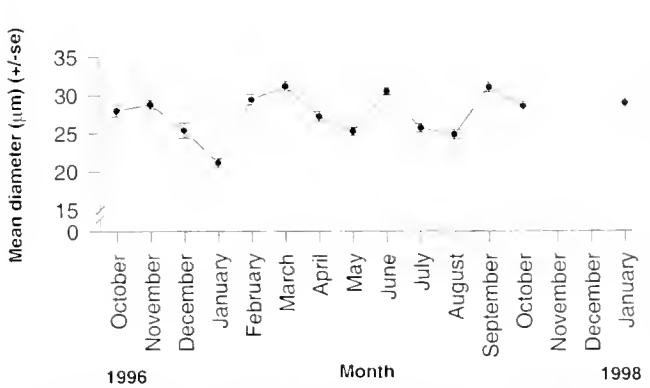

Figure 4. Mean monthly oocyte diameters (±se) from histological analysis of female *R. largillierti* (October 1996–January 1998); n = 10–17 individuals.

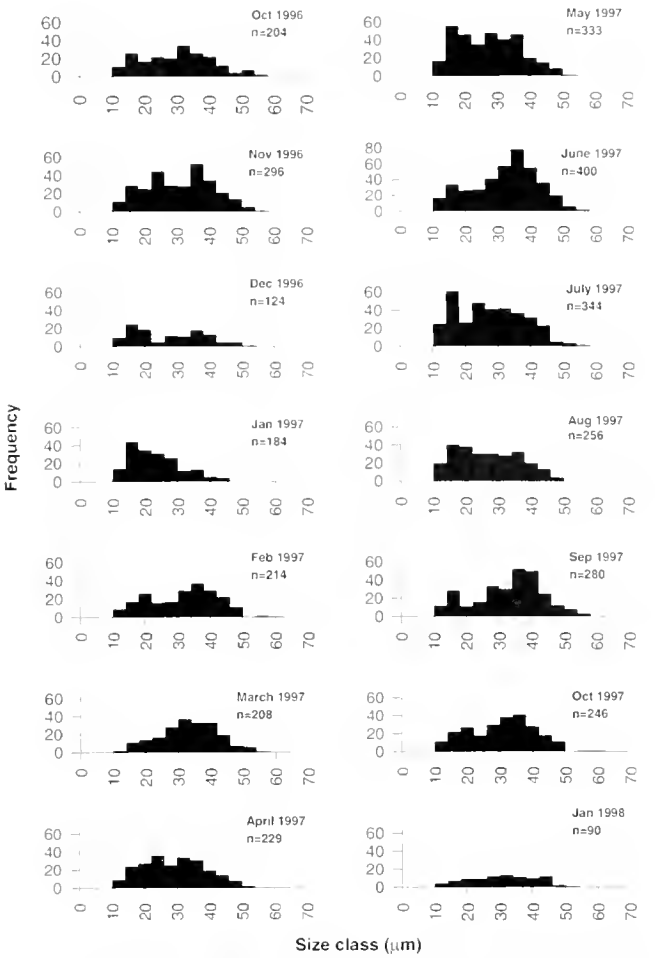

Figure 5. Frequency histograms for all oocyte diameters measured from nine haphazardly selected follicles for each of 10 females from each monthly sample collected for histological analysis (October 1996–January 1998).

August, do not appear to restrict development, as early active, late active and ripe clams were evident as early as July, when water temperatures were at their lowest (13°C; Fig. 3c). *Ruditapes largillierti* occurs throughout New Zealand's waters with its distribution extending to some sub-Antarctic islands (Powell 1979). The Whangateau Harbour is near the northern limit of its distribution. If *R. largillierti* is a species more suited to cooler temperatures, then a period of inactivity following spawning, such as that which occurred from December to March after the 1996/1997 spawning period, may be a result of high summer water temperatures restricting gametogenic development. Early male development, however, commenced in March, soon after the maximum average monthly water temperatures were recorded.

In gonochoristic species, such as the majority of venerid clams, the synchronous development and spawning of gametes in local populations is essential for successful fertilization to occur (Eversole 1989). The fact that small numbers of post-metamorphic clams were found on spat catching bags in Anchor Bay, confirms that spawning and successful fertilization occurred prior to January 1998. Despite successful fertilization occurring, there were slight differences in the timing and duration of development, maturation and spawning between the two sexes. Female reproductive development began later than in males, possibly because the energetic

TABLE 2.

Numbers of male and female *R. largillierti*, and range and mean (±s.d.) of lengths (mm) for clams collected during monthly sampling in the Whangateau Harbour (n = 30). χ^2 values test for sex ratios differing from 1:1 ($\chi^2_1 = 3.84$ at $\alpha = 0.05$).

Month	Males	Females	Length (mm)	Mean (±s.d.)	*P* value
October 1996	20	10	29–56	44.2 (±7.8)	0.068
November 1996	13	17	45–57	50.9 (±2.8)	0.465
December 1996	18	12	43–54	50.4 (±2.9)	0.273
January 1997	13	17	45–53	50.7 (±1.9)	0.465
February 1997	15	15	39–57	47.9 (±4.8)	1.000
March 1997	15	15	33–53	46.7 (±5.1)	1.000
April 1997	16	14	34–58	47.4 (±6.2)	0.715
May 1997	14	16	37–54	48.4 (±3.9)	0.715
June 1997	15	15	34–54	48.3 (±4.0)	1.000
July 1997	14	16	42–54	48.8 (±2.9)	0.715
August 1997	16	14	32–55	48.0 (±5.7)	0.715
September 1997	14	16	36–56	46.9 (±4.3)	0.715
October 1997	15	15	29–53	46.9 (±6.1)	1.000
January 1998	15	15	35–56	47.5 (±4.5)	1.000
Total	213	207			0.761

costs of reproduction are often greater for females than males (Ropes et al. 1984). Therefore, following a major spawning event, females may need to accumulate greater energy reserves relative to males in order to produce gametes once the costs of growth and respiration have been met. However, female gametes develop more quickly through the early active to the late active stage, whereas males develop steadily with a long period dominated by the early active stage of the gametogenic cycle. A pattern of similar overall development with subtle differences between males and females is well documented for many species of clams (e.g., Porter 1974, Manzi et al. 1985, Corni et al. 1985, Hefferman et al. 1989, Hesselman et al. 1989, Rowell et al. 1990, Sbrenna & Campioni 1994).

The appearance of a large number of spawned and partially spawned clams in both October 1996 and 1997 suggests that reproduction followed an annual cycle. Although samples were not collected during November and December 1997, by January 1998 all clams had completely spawned. This was also observed in January 1997. The small number of unspawned late active and ripe individuals that occurred in October 1997 but not in October 1996 are most likely due to inter-annual variability in reproductive development.

Eversole (1989) suggests that comparisons of reproductive development among species would be easier if more quantitative measures of gonadal development were used. Both Kanti et al. (1993) and Villalejo-Fuerte et al. (1996) found a close relationship between oocyte diameters and the gametogenic cycle in *Spisula solidissima similis* in California, and the cockle, *Laevicardium elatum*, in Mexico, respectively. Xie and Burnell (1994) also found that oocyte frequency histograms appeared to support qualitative data on reproductive cycles for both *Tapes philippinarum* and *T. decussatus* on the south coast of Ireland. In general, studies that have employed quantitative analyses indicate that periods of maturation and spawning tend to coincide with maximum oocyte diameter values. However, the association between oocyte diameters and the remainder of the reproductive cycle (excluding spawning events) is unclear (e.g., Eversole et al. 1980, Heffernan & Walker 1989, Hesselman et al. 1989). This also appears true for *R. largillierti*. Peak mean oocyte diameters are associated with months containing eggs in later developmental stages (e.g., October and November 1996, and June and September 1997), and troughs with the presence of higher numbers of primary oocytes (e.g., January, May and August 1997). Although monthly mean oocyte diameters support the findings of qualitative stagings, the patterns are not strong enough to be used on their own as a quantitative indicator of gonad state. Much of this can be attributed to the inherent variability in gonadal state both within and among individuals collected from the same sample, evidenced from the large size range of oocyte diameters that exists for each monthly sample (Fig. 5). Several authors have tried to resolve this problem by reducing the number of stages used to assess gonadal state (e.g., Keck et al. 1975, Eversole et al. 1980).

With the exception of the hardshell clam, *Mercenaria mercenaria*, sex ratios in venerid clams rarely differ significantly from a ratio of 1:1 (Eversole 1989); *M. mercenaria* is protandric with males dominating juvenile stages until sexual maturity is reached. However, the sex-ratios of adults are equal. Overall, this study showed equal numbers of adult male and female clams. However, the restricted size range sampled (only clams over 29 mm) excludes investigation of sex-ratios of juveniles. The sex-ratio of 1 male to 2.33 female clams in the lower end of the sample (29–35 mm) indicates that the ratio may be quite different for smaller clams. However, this is probably more a reflection of the small sample size rather than an indicator of any real trend. The lack of small size classes also makes it difficult to determine the size at which *R. largillierti* reaches sexual maturity. However, all clams between 29–35 mm in length were mature and contained gametes. Analysis of tag and recapture data and experimental growth rates (Gribben 1998) suggest that *R. largillierti* 29–35 mm in length are in their second year of growth. It is likely that sexual maturity is reached between 25–28 mm, as clams grown under experimental culture conditions grew to a maximum size of 24 mm in one year and contained no gametes (Gribben 1998).

The commercial culture of venerid clams is well developed overseas with several species (e.g., *M. mercenaria* and *T. philippinarum*) relying on the production of hatchery reared spat (Manzi

TABLE 3.

Range and mean length (±s.d.) (mm) for male and female *R. largillierti* collected monthly (n = 30) from the Whangateau Harbour, from October 1996 to January 1997.

Month	Male Range (mm)	Female Range (mm)	Males Mean Length (±s.d.) (mm)	Females Mean Length (±s.d.) (mm)
October 1996	29–54	32–56	44.3 (±7.51)	44.0 (±9.4)
November 1996	45–54	46–57	50.7 (±2.7)	51.1 (±2.9)
December 1996	46–53	43–54	50.2 (±2.7)	50.8 (±3.28)
January 1997	48–53	45–52	51.3 (±1.5)	50.2 (±2.0)
February 1997	48–57	39–53	48.6 (±5.3)	47.1 (±4.4)
March 1997	33–53	40–53	46.9 (±5.9)	46.6 (±4.4)
April 1997	34–55	39–58	47.3 (±6.7)	47.6 (±5.7)
May 1997	37–54	43–52	48.2 (±4.9)	48.5 (±3.0)
June 1997	34–53	43–54	47.3 (±4.8)	49.3 (±2.9)
July 1997	43–53	42–54	49 (±2.7)	48.6 (±3.2)
August 1997	32–55	32–54	48.5 (±5.3)	47.5 (±6.3)
September 1997	36–56	42–55	46.9 (±4.7)	46.9 (±3.9)
October 1997	32–53	29–53	44.9 (±5.1)	45.5 (±4.6)
January 1998	35–56	41–54	48.3 (±5.4)	48.5 (±6.2)

& Castagna 1989). Kent et al. (1999) have successfully produced an ongrown hatchery-reared *R. largillierti* spat in Australia. Given that *R. largillierti* spat can be produced in a hatchery, and Gribben (1998) has achieved good growth rates ongrowing juvenile *R. largillierti* intertidally and subtidally in mesh bags on racks, suggests that this species may be amenable to aquaculture.

The results from our research suggest *R. largillierti* in the Whangateau Harbour are ripest from early spring to early summer. Collecting broodstock during this time will maximize egg viability and fertilization success: two of the most important issues facing aquaculturists attempting to produce commercial quantities of spat.

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STOCK ASSESSMENT FOR VENUS CLAM, *CHIONE CALIFORNIENSIS* (BRODERIP, 1835) IN ENSENADA DE LA PAZ, BAJA CALIFORNIA SUR, MÉXICO

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ABSTRACT The stock assessment of the venus clam *Chione californiensis* from Ensenada de La Paz, Baja California Sur, México was made based on survey and commercial catch data. Stock is exploited twice a year, February to early April, and September and October. Elefan I was used to estimate growth parameters of the von Bertalanffy equation. Age structure was computed from a length-age key probability matrix to assign age to length. Natural mortality varying with age and time was estimated using an algorithm based on the Leslie transition matrix and data from unexploited periods. Catchability-at-age was also estimated through an analogous algorithm, but using data for exploited periods. Both algorithms have a numerical solution which uses a least square as fitting criterion. A modified age-structured virtual population analysis (VPA), accounting fishing-effort data, was used to estimate fishing mortality and stock size. Higher values for harvest rate (HR) and fishing mortality (F) occurred during the second fishing period, reaching values of HR = 70%. This is particularly important because it coincides with spawning and recruitment seasons such that exploitation is strongly impacting stock size. Our results also offer an explanation about why clam stocks have been depleted or collapsed.

KEY WORDS: stock assessment, venus clam, *Chione californiensis*, natural mortality-at-age, catchability-at-age, stock depletion

INTRODUCTION

Along the southern coasts of the Peninsula of Baja California there are a variety of clam species that have been exploited for many years. In 1995 the total yield amounted to 9,140 tons, representing 70% of the total clams caught in Mexico. Eight species of Veneridae are exploited in Baja California Sur as target species: *Tivela stultorum* (almeja pismo and pismo clam), *Megapitaria squalida* (almeja chocolata and squalid callista), *M. aurantica* (almeja chocolata roja and golden callista), *Dosinia ponderosa* (almeja blanca and ponderous dosinia), *Peryglipta multicostata* (almeja roñosa de risco and many-ridged venus), *Chione undatela* (frilled californian venus), *C. gnidia* (ornate venus), and *C. californiensis* (almeja roñosa and californian venus clam) (Holguín 1976, Baqueiro et al. 1982, Baqueiro & Guajardo 1984, Prado-Ancona 1998).

The venus clam *C. californiensis* is an intertidal bivalve distributed from the coastline up to 69 m depth, in sandy bottoms. In Ensenada de La Paz (Fig. 1), the highest densities of about 48 clams m^{-2} have been observed in the upper portion of the mid-intertidal area between 0 and 3 m deep (García-Domínguez et al. 1994). Distribution is determined by the type of bottom, with the clam living on sandy or rocky-sandy bottoms (García-Domínguez 1991). Catch takes place in shallow waters during low tides, when fishermen collect clams by hand or using some dredging instruments. In several locations along the southern Peninsula of Baja California, clam stocks have decreased and some have disappeared almost completely (García-Domínguez 1991). There are two apparent reasons for this: they have been overexploited because of their high vulnerability and fisheries operate under an open access scheme, or because of bottom disturbances caused by human activities along the coasts. For both, the absence of knowledge to support appropriate management measures has been an additional problem. In this paper, the stock assessment of a venus clam *C. californiensis* in Ensenada de La Paz is developed as a contribution to the knowledge of the population dynamics. Some management aspects are discussed.

MATERIAL AND METHODS

Ensenada de La Paz has an area of 45 km^2, with a communication with Bahia de La Paz through a channel 1.5 km long and 4 km wide (Fig. 1). The stock we studied is located in a place known as Las Palmitas (24°10′N and 110°24′W). Samples were taken from July 29, 1988 to September 20, 1989 on a transect perpendicular to the shoreline, passing across the high clam-density area. Venus clams were collected every 25 m along the transect, where 20 cm^3 of sediment (a square of 1 m per side and a depth of 20 cm) was taken and sieved through a 1-mm mesh. The total length of the clams was measured with a precision of 0.01 mm within a range of 4 to 43.5 mm. A number of clams were also weighed to compute the length-weight relationship.

The venus clam stock is exploited twice a year, within the period of time we studied, September to October 1988 and February to April 1989. Catch records were collected from Sepesca (1988, 1989) for these periods.

Growth

The ELEFAN I method (Pauly and David 1981, Pauly 1987) was used to estimate the parameters of the von Bertalanffy growth equation (VBGE). For this, length frequency distributions were grouped in 3-mm length-class sizes.

Age Structure

Age structure was estimated as follows. First, we selected the Gaussian components in the length-frequency distributions, using the Bhattacharya (1967) method, to obtain mean length and variance for some age groups. Then a function of how variance of the mean length-at-age group changes with relative age was fitted. The relative age from modal lengths was estimated according to the VBGE. With this function we were able to estimate mean and variance for all ages in the stock. Normal distributions representing age groups were converted to a standardized normal distribution $N(0,\sigma^2)$ to obtain a length-age key in terms of probability, which

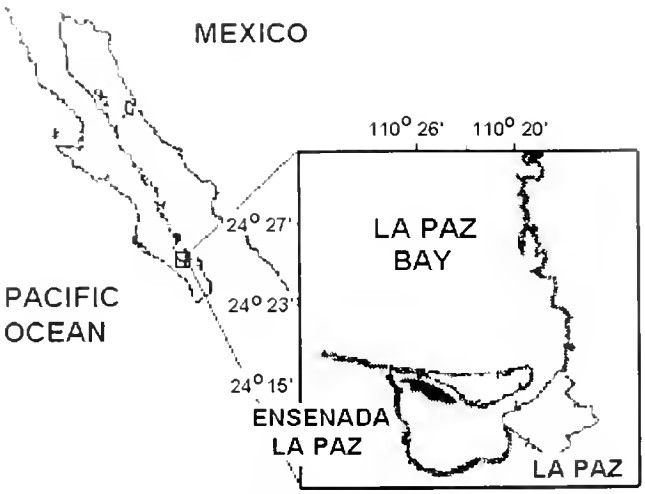

Figure 1. Location of Ensenada de La Paz in Baja California Sur, Mexico.

was used to assign the probability for a given length to belong to a given age. Here, we named it the length-age key probability matrix, PM. Then, the product of the transposed vector of a length frequency distribution and the PM resulted in an age-structured vector.

Natural Mortality-at-Length

Natural mortality, M, was estimated through an iterative process based in the transition matrix following Arreguín-Sánchez et al. (2000). This procedure computes M varying with length and time, and is described by the relationship

$$N(\ell, t + \Delta t) = \Sigma_k[G(\ell, k) \times S(k)] \times N(k, t) \quad (1)$$

where N represents the length structure vectors, in this case expressed as stock density, and k and ℓ represent successive length classes. G is the growth matrix whose elements represent growth probabilities per length class according to the VBGE (following Shepherd 1987). S is the survival matrix, whose elements represent an average survival estimate of the k length class, and t represents time. Because the venus clam is a relatively short-lived species, age was measured in months; Δt represents time between samples. When $\Delta t \neq 1$ mo, time was measured as a proportion, taking the month as a unit. Elements in the survival matrix are expressed as $S(k) = \exp(-M)\Delta t$ when there is no fishing, and $S(k) = \exp(-(M + F))\ \Delta t$ when exploitation is being done, with F being the instantaneous rate of fishing mortality.

Because exploitation occurred in two periods, September and October and February to March, Eq. 1 was iteratively solved for those months when there was no fishing. For this, the growth matrix was computed using the estimated parameters of the VBGE; $N(k, t)$ and $N(\ell, t + \Delta t)$ are known, and M was varied. Fitting process used a least squares algorithm with the form

$$\text{MIN} \sum_{\ell}^{n} (N_{obs}(\ell, t + \Delta t) - N_{est}(\ell, t = \Delta t))^2 \quad (2)$$

Catchability-at-Length

The average yield per fishermen in a regular fishing season is about 200 clams per hour, corresponding to an average density of 33.5 clams m^{-2} in a searching area of 10 km^2. This information provides an index of fishing effort at time t, E_t. Catchability was estimated following Arreguín-Sánchez (1996) and Arreguín-Sánchez and Pitcher (1999), whose method is also based on Eq. 1, but using catch per unit effort length frequency data for the exploited periods, but giving the M-at-length values estimated as above. In such case, survival is expressed as $s(k) = \exp - [M(k, t) + q(k, t)f(t)]$, with q and f the catchability coefficient and fishing effort, respectively. Then, iterations were done varying catchability coefficients in elements of the survival matrix. The fitting process also used a least-square algorithm as in Eq. 2.

M-at-length and catchability-at-length were easily transformed to age by computing relative ages through the VBGE.

Stock Size and Fishing Mortality

Stock size was estimated through the use of the Gulland (1965) and Murphy (1965) methods as follows:

$$\text{Let} \quad C_{a,t} = N_{a,t} \times E_{a,t} \quad (3a)$$

where

$$N_{a,t} = N_{a,t-1} \times \exp - (M_{a,t} + F_{a,t}) \quad (3b)$$

which represents the stock survival process for two successive ages. $E_{a,t}$ represents the exploitation rate-at-age, with a the index for age, and $E_{a,t}$ expressed by

$$E_{a,t} = \left(\frac{F_{a,t}}{(M_{a,t} + F_{a,t})}\right)\{1 - \exp(-[M_{a,t} + F_{a,t}])\} \quad (4)$$

The ratio of catch at successive times is represented by

$$\frac{C_{a}+1, t+1}{C_{a,t}} = R_{a,t} = \frac{\{\exp[-(M_{a,t} + F_{a,t}])\} \times E_{a+1, t+1}}{E_{a,t}} \quad (5)$$

Given M_t and F_t, a forward solution can be obtained for two situations.

$$\text{For } E_{a,t} \neq 0, \quad E_{a+1,t+1} = R_{a,t} \times E_{a,t} \times \exp(-[M_{a,t} + F_{a,t}]) \quad (6a)$$

and

$$\text{For } E_{a,t} = 0, \quad E_{a+1,t+1} = R_{a-x,t-x} \times E_{a-x,t-x} \times \exp\{-([t-x]M_{a-x,t-x} + F_{a-x,t-x})\} \quad (6b)$$

Where $(a - x)$ and $(t - x)$ represent age and time between the exploited age at time t and the nonexploited age at time x.

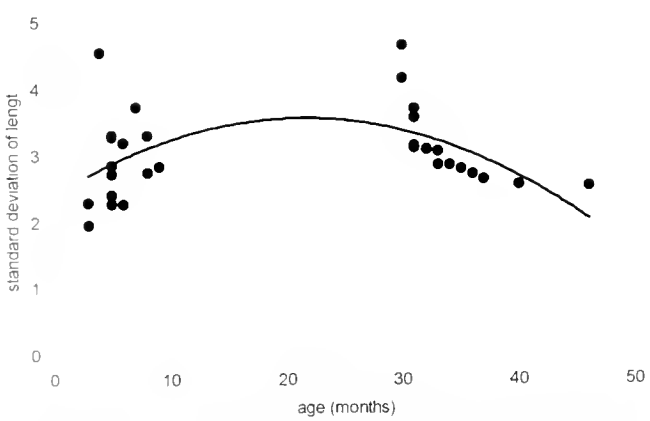

Figure 2. Standard deviation(s) of length (millimeter) per age group, as a function of age (months) for the venus clam *C. californiensis* from Ensenada de La Paz, Mexico.

Once estimates for $E_{a,t}$ are obtained, an estimate for $N_{a,t}$ for the last age group can be obtained from Eq. 3a, and for other age groups from Eq. 3b. In this case, the fitting process was a least-squares algorithm based on catch with the form

$$\text{MIN} \sum_{a=1}^{a_{max}} [C_{obs}(a,t+\Delta t) - C_{est}(a,t+\Delta t)] \quad (7)$$

where C_{obs} = observed catch and C_{est} = estimated catch, which was obtained from Eq. 3 (with estimated values for $N_{a,t}$ and $E_{a,t}$).

Murphy (1965) suggested a forward solution to a catch equation in a cohort analysis; for this, an estimate of recruits should be available. Here, recruits per month were estimated from survey and commercial catch as follows. During catch periods, Eq. 6a was applied using a seed value for $F_{a,t}$ as in the virtual population analysis (Gulland 1965, Gulland 1983, Pope 1972, Pope & Shepherd 1982, Pope & Shepherd 1985), and using Eq. 7 as the fitting criterion. Results were compared with survey abundance data, and the average ratio was used to estimate recruitment over those months with no fishing. Once recruits were obtained, the stock assessment process, following Murphy's algorithm, was developed.

RESULTS

Growth

Growth parameters for the von Bertalanffy growth equation were $L\infty$ = 48.2 mm, K = 0.735 year^{-1}, and to = −0.64 years. Parameters for the length (millimeters) - weight (grams) relationship were a = 0.00016 and the slope b = 3.234. These estimates resulted in a value for asymptotic weight of $W\infty$ = 46 g.

Age Structure

Because the venus clam is a relatively short-lived species, with a life span of around 4 y (Castro-Ortiz & García-Domínguez 1993), we decided to express age in month units. Once Gaussian components were obtained from length-frequency data, a plot representing variance per age group as a function of age was approached as a parabolic function (Fig. 2), which was used to estimate variance-at-age and for the construction of the PM and the age structure, as described above.

M-at-Length

The algorithm used to estimate M-at-length provided values for those ages and months where fishing was closed (Table 1), where stock declination is only from natural causes. In general, M-at-age declines with age, from an average of $M_{a,t}$ = 1.48 mo^{-1} at ages between 1 and 6–7 mo, to $M_{a,t}$ = 1–15 mo^{-1} for clams around 20–24 mo old (Fig. 3a). A time variation was also observed that suggested a seasonal behavior (Fig. 3b) with higher values after mid-year. $M_{a,t}$ estimates for exploited periods were obtained interpolating values from tendency over time.

Catchability-at-Length

Catchability patterns with length have a similar shape, but a different magnitude between the two catch periods (Fig. 4), with

TABLE 1.

Natural mortality-at-age and time in years (indicated by column heads as proportion of the year) for the venus clam *C. californiensis* from Ensenada de La Paz, México. Estimation follows Arreguín-Sánchez et al. (unpubl. data).

Age (Months)	0.578	0.682	0.726	0.786	0.841	0.874	0.956	0.025	0.082	0.118	0.189	0.260	0.304	0.463	0.548
1	3.438	2.891	2.308	1.603	1.215	1.113	1.411	1.538	1.825	1.567	1.036	0.847	0.888	2.139	3.223
2	2.928	2.435	1.983	1.447	1.157	1.086	1.312	1.423	1.603	1.363	0.911	0.766	0.815	1.927	2.783
3	2.678	2.213	1.821	1.366	1.126	1.071	1.259	1.362	1.491	1.260	0.848	0.724	0.777	1.819	2.565
4	2.520	2.073	1.719	1.314	1.105	1.060	1.225	1.323	1.419	1.195	0.807	0.697	0.752	1.748	2.426
5	2.407	1.975	1.646	1.276	1.090	1.053	1.200	1.294	1.368	1.148	0.778	0.677	0.734	1.697	2.326
6	2.322	1.900	1.591	1.247	1.078	1.047	1.180	1.272	1.329	1.113	0.756	0.662	0.719	1.657	2.251
7	2.255	1.841	1.547	1.223	1.068	1.042	1.164	1.254	1.297	1.085	0.739	0.650	0.708	1.626	2.191
8	2.200	1.793	1.511	1.204	1.060	1.038	1.151	1.239	1.272	1.062	0.724	0.640	0.699	1.600	2.142
9	2.154	1.753	1.481	1.188	1.053	1.035	1.140	1.226	1.250	1.042	0.712	0.632	0.691	1.578	2.101
10	2.115	1.719	1.456	1.174	1.047	1.032	1.131	1.215	1.232	1.026	0.702	0.625	0.684	1.560	2.067
11	2.082	1.690	1.434	1.162	1.042	1.029	1.123	1.206	1.216	1.012	0.693	0.619	0.678	1.544	2.037
12	2.053	1.665	1.415	1.152	1.038	1.027	1.116	1.198	1.203	0.999	0.685	0.613	0.673	1.530	2.011
13	2.028	1.643	1.399	1.143	1.034	1.025	1.109	1.191	1.191	0.989	0.679	0.608	0.669	1.518	1.988
14	2.005	1.624	1.384	1.135	1.030	1.023	1.104	1.184	1.180	0.979	0.673	0.604	0.665	1.507	1.968
15	1.985	1.607	1.371	1.127	1.027	1.022	1.099	1.179	1.170	0.971	0.667	0.600	0.661	1.497	1.950
16	1.968	1.591	1.359	1.121	1.024	1.020	1.094	1.173	1.162	0.963	0.662	0.597	0.658	1.488	1.934
17	1.951	1.577	1.349	1.115	1.022	1.019	1.090	1.169	1.154	0.956	0.658	0.594	0.655	1.480	1.920
18	1.937	1.565	1.339	1.110	1.019	1.018	1.086	1.165	1.147	0.950	0.654	0.591	0.653	1.473	1.907
19	1.924	1.553	1.331	1.105	1.017	1.017	1.083	1.161	1.141	0.944	0.651	0.589	0.650	1.467	1.895
20	1.912	1.543	1.323	1.101	1.015	1.016	1.080	1.157	1.135	0.939	0.647	0.586	0.648	1.461	1.884
21	1.901	1.534	1.316	1.097	1.013	1.015	1.077	1.154	1.130	0.935	0.644	0.584	0.646	1.455	1.874
22	1.891	1.525	1.309	1.093	1.012	1.014	1.075	1.151	1.125	0.930	0.642	0.582	0.644	1.450	1.865
23	1.882	1.517	1.303	1.089	1.010	1.013	1.072	1.149	1.121	0.926	0.639	0.581	0.643	1.446	1.857
24	1.874	1.510	1.298	1.086	1.009	1.013	1.070	1.146	1.117	0.923	0.637	0.579	0.641	1.442	1.850
Average	2.184	1.781	1.500	1.195	1.055	1.035	1.144	1.230	1.262	1.053	0.719	0.635	0.694	1.588	2.126
Standard deviation	0.381	0.336	0.247	0.128	0.052	0.025	0.085	0.098	0.174	0.158	0.098	0.066	0.062	0.173	0.336
CV	0.175	0.188	0.165	0.107	0.049	0.025	0.074	0.079	0.138	0.150	0.136	0.104	0.089	0.109	0.158

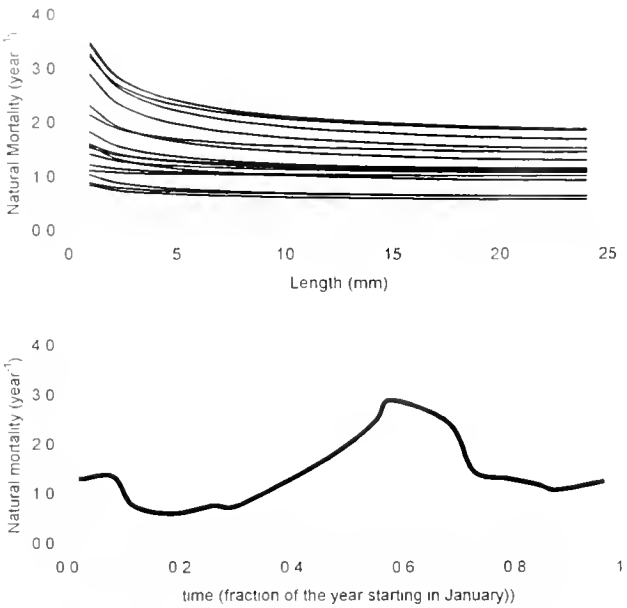

Figure 3. Natural mortality as a function of length (top) and time (bottom) for the venus clam *C. californiensis* from Ensenada de La Paz, Mexico.

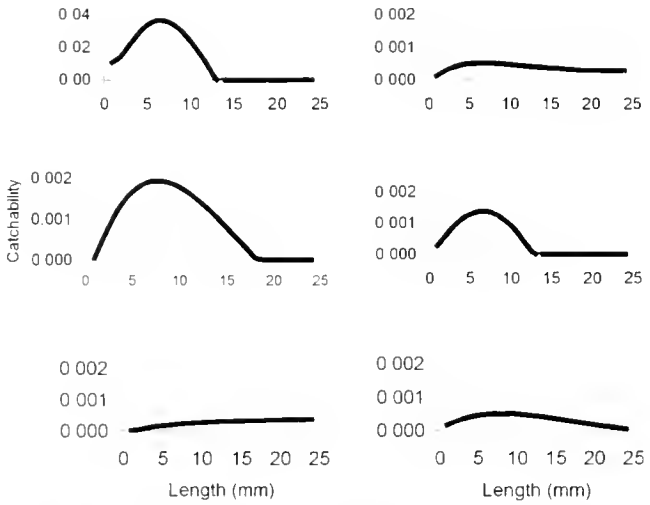

Figure 4. Catchability-at-length patterns estimated for the two fishing periods for the venus clam *C. californiensis* in Ensenada de La Paz, Mexico. Note that catchability scale for all plots is equal with exception of February (top left) where catchability is higher by one order of magnitude.

higher values for clams aged 7–8 mo. In general, catchability-at-length values were lower during September and October where relatively small values were found for clams between 4 and 12 mo, and ranging from q = 0.0003 to q = 0.00137. Higher catchability values were found during the first fishing period of the year, particularly in February where catchability varied between 0.008 to 0.036 for clams between 2 and 12 mo. Catchability decrease later during March and early April in one order of magnitude (Table 2). The main feature of catchability-at-length patterns is that catchability values for the venus clam are considerably higher for adults just before spawning (García-Domínguez & Levy-Pérez 1995).

TABLE 2.

Catchability-at-length estimated for the venus clam *C. californiensis* from Ensenada de La Paz, México. Estimation follows Arreguín-Sánchez (1996), Arreguín-Sánchez and Pitcher (1999), and Martínez-Aguilar et al. (1999). (Column heads represent dates as proportion of the year.)

Age (Months)	0.12	0.19	0.26	0.68	0.73	0.79
1	0.010809	0.000051	0.000001	0.000116	0.000275	0.000156
2	0.014626	0.000584	0.000041	0.000278	0.000592	0.000252
3	0.021525	0.001038	0.000093	0.000388	0.000879	0.000331
4	0.028372	0.001399	0.000135	0.000459	0.001112	0.000393
5	0.033518	0.001664	0.000169	0.000499	0.001275	0.000439
6	0.036246	0.001837	0.000197	0.000518	0.001362	0.000470
7	0.036406	0.001924	0.000220	0.000520	0.001368	0.000487
8	0.034173	0.001937	0.000239	0.000512	0.001294	0.000493
9	0.029886	0.001885	0.000255	0.000496	0.001144	0.000488
10	0.023948	0.001780	0.000269	0.000476	0.000921	0.000476
11	0.016771	0.001632	0.000280	0.000454	0.000633	0.000457
12	0.008736	0.001450	0.000291	0.000431	0.000286	0.000432
13	0.000177	0.001242	0.000300	0.000408	0.000001	0.000404
14	0.000001	0.001017	0.000308	0.000387	0.000001	0.000372
15	0.000001	0.000780	0.000316	0.000367	0.000001	0.000338
16	0.000001	0.000536	0.000323	0.000349	0.000001	0.000303
17	0.000001	0.000290	0.000330	0.000334	0.000001	0.000267
18	0.000001	0.000046	0.000337	0.000321	0.000001	0.000231
19	0.000001	0.000001	0.000343	0.000310	0.000001	0.000195
20	0.000001	0.000001	0.000349	0.000301	0.000001	0.000159
21	0.000001	0.000001	0.000355	0.000295	0.000001	0.000124
22	0.000001	0.000001	0.000360	0.000290	0.000001	0.000090
23	0.000001	0.000001	0.000366	0.000288	0.000001	0.000058
24	0.000001	0.000001	0.000371	0.000287	0.000001	0.000026

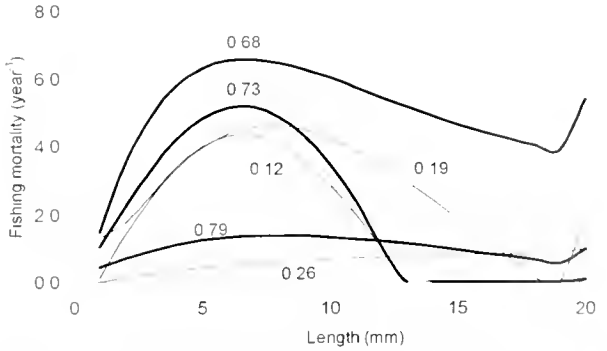

Figure 5. Fishing mortality-at-length patterns estimated for the venus clam *C. californiensis* in Ensenada de La Paz. Thin lines correspond to the period of February to early April, while bold lines are for September and October.

Fishing Mortality and Stock Size

Fishing mortality-at-age, F_a, shows a similar pattern as catchability, with higher values for clams age 6–8 months (Fig. 5), during September ($F_2 = 6.51$ mo^{-1}) and October ($F_2 = 5.04$ mo^{-1}; Table 2). García-Domínguez and Levy-Pérez (1995) reported a reproductive period from April to December with a peak from August to November. These high values of fishing mortality impact spawning stock during the peak of the reproductive season.

Even though there are two spawning seasons, stock size estimation (Table 3) indicates that dynamics are strongly governed by one strong cohort that is recruited during September and October: remaining more time in the stock than the early Spring cohort. Stock size was over 60 million clams per year, whereas catch amounted to approximately 5 million clams. Average annual harvest rate ($HR = \frac{C}{N}$) was 14%, but in September it reached a value of 0.8%. The average exploitation rate, $\frac{F}{Z}$, varied from 0.45 to 0.47 between fishing periods, but with $\frac{F}{Z} = 0.72$ in September. The average proportion of the stock under exploitation, $(\frac{F}{Z})(1 - e^{-Z})$, was 0.43, but in September it reached 0.72.

DISCUSSION

Most clam stocks are highly vulnerable to fishing and to habitat degradation. Many stocks are located in shallow waters, and species generally are associated with specific habitats. Exploitation of these stocks require special attention by managers, users, and scientists if sustainable yields are expected.

For growth estimates, length-based methods have been used for bivalves with success as shown by Vakily (1990) and Defeo et al. (1992). *C. californiensis* is a relatively short-lived species, with a lifespan of about 3 to 4 y.

M, as estimated here, indicates a decreasing pattern with age, as occurred with other clam stocks (McLachlan et al. 1996). For the clam, this behavior is expected because of density-dependent effects such as space competition during settlement and food competition once individuals have settled. M of adults decreased slowly because they have some advantages over small individuals for space and feeding. McLachlan et al. (1996) analyzed 15 clam fisheries around the world and suggested that the unharvested fraction of exploited sandy-beach stocks may be directly related to the amount of fishing because of sediment disturbance and incidental damage during the collection. For the venus clam, higher values of fishing mortality were present during September, coinciding with high values of M, as expressed by the seasonal pattern. In this case, however, higher values for M also coincide with the peak of the spawning period.

Catchability shows a large variation with size and time, representing a large variation in vulnerability to fishing. Results suggest higher catchability values are present during the prereproductive and reproductive seasons. This is of importance because great vulnerability to fishing can negatively impact the reproductive success and, together with M, it could be a potential cause for a collapsing stock. Fishing mortality reflects catchability variations, with higher values for young adults than for older clams. McLachlan et al. (1996) suggests M be directly related to stock density. For the venus clam, this is not clear, but instead, M appears to be related to spawning and the recruitment process.

Stock size estimates and the dynamics described in previous paragraphs suggest there is a dominant cohort during the year, and the population dynamics, fishing success, and yields will depend upon the size and survival of such cohort. The first aspect about stock size is that even though *C. californiensis* has a longevity of 3 to 4 y, dominant cohort practically disappears after 1 y. Survival after 1 y resulted in a poor number of individuals (less than 1%). This condition of the stock suggests that current exploitation is

TABLE 3.

Stock-size estimation (millions) for *C. californiensis* from Ensenada de La Paz, México, during the first year of life of the cohorts (see text for explanation).

Age (Months)	0.58	0.68	0.73	0.79	0.84	0.87	0.96	0.02	0.08	0.12	0.19	0.26	0.30	0.46	0.55
1	18.138	9.043	0.180	0.183	28.666	0.041	0.015	0.018	0.037	0.031	0.335	21.942	0.138	0.083	0.558
2	23.505	8.566	0.912	1.209	0.097	8.646	0.016	0.006	0.009	0.030	0.237	4.208	4.367	0.056	0.025
3	15.327	5.037	1.742	2.357	0.683	0.031	3.543	0.007	0.003	0.033	0.470	4.743	0.977	1.903	0.019
4	9.500	3.746	2.448	3.322	1.375	0.225	0.013	1.485	0.004	0.044	0.773	5.618	1.192	0.443	0.689
5	4.489	2.524	2.308	3.174	1.978	0.462	0.094	0.006	0.844	0.045	0.931	5.143	1.487	0.554	0.167
6	1.577	1.265	1.526	2.163	1.919	0.675	0.195	0.041	0.003	0.035	0.858	3.685	1.413	0.704	0.215
7	0.438	0.465	0.778	1.141	1.322	0.663	0.286	0.086	0.024	0.022	0.642	2.210	1.042	0.679	0.279
8	0.108	0.134	0.342	0.503	0.704	0.461	0.281	0.128	0.050	0.013	0.410	1.176	0.640	0.506	0.274
9	0.027	0.034	0.143	0.196	0.313	0.248	0.197	0.127	0.075	0.007	0.234	0.579	0.347	0.314	0.207
10	0.007	0.009	0.062	0.072	0.123	0.111	0.106	0.089	0.075	0.004	0.126	0.272	0.174	0.172	0.130
11	0.002	0.002	0.030	0.029	0.046	0.044	0.047	0.048	0.053	0.002	0.065	0.124	0.083	0.086	0.072
12	0.001	0.001	0.020	0.016	0.018	0.016	0.019	0.022	0.029	0.001	0.033	0.056	0.038	0.041	0.036

done on young and prereproductive clams and it is probably not the best choice to sustain the fishery. These facts also contribute to make the venus clam an extremely fragile stock in those cases when the fishery is developed under an open access scheme or a poorly controlled exploitation, as commonly occurs with many artisanal fisheries.

These types of problems have been experienced by other clam species. Gallucci (1982) studied the fishery of *Spisula solidissima*, where the rate of loss of biomass was greater than the stock growth rate. Sasaki (1993) evaluated the state of exploitation for the *S. sachalinensis* fishery and showed that stock abundance varied strongly during the year, making this an unstable fishery. Defeo (1989) reported a depleted open-access fishery of *Mesodesma mactroides* in Uruguay that was temporally closed and reopened under a controlled management scheme after it recovered. Tarr (1994) also reported a declining fishery on the white sand mussel *Donax serra* in southern Africa, where even though several measures to control open access were attempted since 1968, all licenses were finally revoked. In Weave Bay, New Zealand, McLachlan et al. (1996) also reported a depleted stock for the toheroa clam *Paphies ventricosa*, which has not recovered despite efforts made over 20 y.

Experience with several beach clams exploited in the intertidal areas shows they are highly vulnerable to harvest because of the low costs involved in their exploitation. Additionally, loss of habitat because of human activities increases mortality. All of these aspects must be considered by managers in order to sustain fisheries. For *C. californiensis*, we also showed that highest mortality (both fishing and natural) occurred during reproduction and spawning seasons. This is an important impact on the stock, particularly under high fishing pressure in an open-access fishery.

Previous experience with clams in Bahia de La Paz and adjacent regions indicate that the lack of knowledge of population dynamics as described here was a large contributor to the decline of clam stocks. This report attempts to advise fishery managers about the relevant aspects of the population biology of the venus clam to maintain biomass and, if possible, current yields. Our advice is that this type of analysis must be made for the most recent years as a basis to simulate experiments resulting in management scenarios.

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MODELING GROWTH OF THE NORTHERN QUAHOG, *MERCENARIA MERCENARIA*

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ABSTRACT Growth of the northern quahog, *Mercenaria mercenaria* (Linné), was deterministically modeled over the life span of the bivalve as well as during its first growing season (nursery stage) in Rhode Island waters. Specifically, the von Bertalanffy growth equation was used to predict increases in shell length (millimeters), weight (grams), and the relative growth rate (% increase in weight or volume per day) at various instantaneous annual growth coefficients (K). The relative growth rate (RGR) was also determined by averaging over different time intervals including 1, 4, 7, 14, and 28 days and annually. The age at which the maximum shell length and weight was reached varied with K. A higher K (0.30) resulted in more rapid initial growth and an earlier asymptote, while a lower K (0.20 and 0.10) resulted in slower initial growth and a later asymptote. RGR averaged over an annual time interval (annual RGR) decreased rapidly as the northern quahog aged, approaching 0.5% increase/day after age 2. The annual RGR's at different K values were similar, indicating that RGR was relatively insensitive to changes in K. During the first growing season (210 days in the northeast), the increase in shell length predicted by the von Bertalanffy equation was linear with a slope determined by K, that is, a higher K resulted in a greater slope. RGR, however, varied greatly during the first growing season, decreasing from 11% increase/day at 90 days after spawning to 2% increase/day at 210 days after spawning. The RGRs at different K values were also insensitive to changes in K. There were no detectable differences between RGR determinations at a K of 0.10, 0.20, and 0.30 with varying time averaging intervals (T); however, the value of RGR at a specific time varied with the time interval used to calculate RGR. The a and b coefficients estimated for the weight-length relationship from the adult and nursery stage northern quahogs differed from each other, and published measures from Narragansett Bay northern quahogs. This suggests that researchers should use data collected from northern quahogs in a size range similar to that being modeled when estimating biomass from length and abundance data. Predicted lengths and RGRs were compared to observed lengths and RGRs from a field experiment growing northern quahogs in an experimental-scale upweller (nursery stage). Our northern quahogs grew at a K of 0.25 indicating favorable conditions for growth. Early in the experiment (between 70 and 100 days after spawning), the experimental RGR differed markedly from the predicted measure; however, after 100 days post spawning the experimental RGR followed the general trend of decreasing RGR over time, but was higher than predicted.

KEY WORDS: northern quahog, *Mercenaria mercenaria*, growth modelling

INTRODUCTION

The success of a shellfish aquaculture operation depends on optimizing production, specifically, on maximizing growth and survival. Growth is defined as an increase in the size of an individual or the mean increase in the size of a population (Malouf & Bricelj 1989). Growth is usually expressed as a change in shell length, weight, or volume. The change in size (shell length, weight, or volume) per unit time is defined as the growth rate. The growth rate is usually expressed as an absolute growth rate, a relative growth rate, or an instantaneous (specific) growth rate (Ricker 1975). The absolute growth rate describes an increase in size (shell length, weight, or volume) over a specific time interval, usually a month or year. The absolute growth rate does not account for differences in the initial size of the animal. Two northern quahogs may have the same absolute growth rate, but different initial sizes; therefore, the smaller northern quahog is growing more relative to its initial size than the larger northern quahog. To incorporate the effect of initial size on growth, the relative growth rate (RGR) or specific growth rate (SGR) is commonly employed. The RGR measures the change in size of the animal relative to its initial size. The SGR estimates the growth rate (change in weight over time) and then uses a log or natural log transformation (Jobling 1994).

A number of growth models are presented in the literature to describe the size (length or weight) of a northern quahog over time. The von Bertalanffy model is the standard for marine species (King 1995) and has been successfully applied to the northern quahog (Jones et al. 1989). Traditionally, northern quahog growth studies have focused on aging northern quahogs in the natural environment based on sclerochronology or shell growth rings (Kennish & Loveland 1980, Peterson et al. 1984, Jones et al. 1989, Rice et al. 1989, Arnold et al. 1991, Slattery et al. 1991). These studies have documented the effect of environmental conditions (Ansell 1968, Jones et al. 1989), habitat (Peterson et al. 1984, Slattery et al. 1991), and fishing pressure (Rice et al. 1989) on the growth history of the animal. In aquaculture research, sclerochronological measurements are not required for documenting growth because the age is based on time from spawning or settlement.

Growth of the northern quahog varies both temporally and spatially. Ansell (1968) documented growth of the northern quahog over its natural geographic range. Jones et al. (1989) compared Ansell's data to their own and found that northern quahogs from Narragansett Bay grew exceptionally fast during the first 2 years of life. They also reported that growth varied widely throughout the bay. They found that the von Bertalanffy estimates of the maximum shell length (L_∞) varied from 67 to 100 mm, the growth coefficient (K) varied from 0.16 to 0.30, and the time at zero length (t_0) varied from −0.05 to −0.81 years. *Mercenaria mercenaria* have been known to live as long as 40 years, although only 10% of the northern quahogs from Narragansett Bay live longer than 30 years (Jones et al. 1989). Rice et al. (1989) also documented differences in growth throughout the bay. Northern quahogs sampled from Greenwich cove exhibited an L_∞ of 87 mm and a K of 0.09, while northern quahogs from the West Passage exhibited an L_∞ of 111 mm and a K of 0.10. There are a variety of factors that could account for these differences including fishing pressure (density), environmental conditions (temperature, salinity, oxygen, and food availability), sediment type, food concentration and quality, nutrient loading, and current speed.

The diversity of growth estimates used in shellfish aquaculture

research is daunting. Malinowski (1988) described northern quahog growth as an increase in shell length (mm) over the number of days from spawning, an increase in microns per day, as well as a percent weekly increase in packed volume. Manzi et al. (1984) defined northern quahog growth in terms of increases in shell length and settled volume (biomass). Since the time intervals varied between measurements, both authors converted biomass increases into an equivalent monthly relative growth rate. Similarly, a number of studies have transformed biomass increases into daily, weekly, and monthly growth rates (Manzi et al. 1986, Malinowski & Siddall 1989, Hadley et al. 1999). Although the majority of researchers have utilized RGR to quantify northern quahog growth, Rheault and Rice (1996) successfully applied SGR to a shellfish experiment, characterizing oyster and scallop growth rates to varying degrees of food limitation.

The variability present among the above experiments makes it extremely difficult to compare growth rates between studies, because growth is being expressed during different time periods and over varying sizes. Furthermore, investigators have failed to predict or model growth under optimal conditions. More importantly, without an estimate or expectation of growth, researchers lack a baseline for comparison. In other words, observed differences in growth due to experimental treatments could be confounded by differences in the predictable growth of the animal depending on the measure used.

Thus, the purpose of this paper is to:

1. present a set of expectations for northern quahog growth over both a life time and the first season of growth based on measures of shell length, weight, and volume;
2. describe the sensitivity of RGR to changes in the growth coefficient (K) and the time averaging period over which RGR is measured; and
3. to finally compare the theoretical growth models to observations from the field and from a nursery upweller.

MATERIALS AND METHODS

The von Bertalanffy growth model was used to characterize growth of the northern quahog in the coastal waters of Rhode Island. The von Bertalanffy equation in terms of shell length is:

$$L_t = L_\infty[1 - e^{-K(t-t_o)}]$$

where L_t is the shell length at age t, L_∞ is the maximum shell length attained by the animal, K is the instantaneous annual growth coefficient, t is the age in years, and t_o is the theoretical age (years) at zero length (King 1995). Length in centimeters at time t (L_t) was converted to weight in grams at time t (W_t) by using:

$$W_t = aL_t^b$$

where a is a unit conversion factor and b is the volumetric expansion factor. The growth rate was defined as the daily relative growth rate (RGR) and as the specific growth rate (SGR). RGR was calculated as:

$$\text{RGR} = \left(\frac{W_{final} - W_{initial}}{W_{initial}}\right)(T^{-1})$$

where W_{final} is the final weight (g) and $W_{initial}$ is the initial weight (g) and T (days) is the intervening time period (Ricker 1975). The RGR is expressed as a % increase per day. The SGR was calculated as:

$$SGR = \left(\frac{LN(W_{final}) - LN(W_{initial})}{T}\right)(100)$$

where LN is the natural log, W_{final} is the final weight (g), $W_{initial}$ is the initial weight (g), and T (days) is the intervening time period. The SGR is also expressed as a % increase per day.

Growth Over the Lifetime

The increase in the length and weight of the northern quahog was characterized over the course of the bivalve's lifetime at instantaneous annual growth coefficients (K) of 0.10, 0.20, and 0.30. These growth coefficients were selected to encompass the range observed in Narragansett Bay. The maximum age (lifespan) of the northern quahog was assumed to be 40 years (Jones et al. 1989) and t_o was assumed to be +0.10 years or 36 days. A value of +0.10 was chosen because the time from spawning to settlement can take anywhere from three to five weeks depending on water temperature (Rice 1992). The L_∞ for the von Bertalanffy and the a and b weight coefficients for the length-weight relationship were based on samples collected in Narragansett Bay and are summarized in Table 1 (Jones et al. 1989, Rice et al. 1989). The daily RGR and SGR averaged on an annual basis were calculated over the life span of the quahog.

Growth During the First Growing Season

To document growth of the northern quahog during the nursery stage (2 to 14 mm) shell length, weight, and RGR were modeled during the first growing season. In Rhode Island waters, the growing season is approximately seven months (210 days) and lasts from mid April to mid November (Ansell 1968). The model was initiated at the time of spawning and t_o and L_∞ values of 36 days and 14.0 cm were used. The increase in shell length, weight, RGR, and SGR were modeled during the first growing season (36 to 210 days) at K values of 0.10, 0.20, and 0.30. The RGR during the first growing season was further investigated by varying the growth interval (T) between determinations. Specifically, growth intervals of 1, 4, 7, 14, and 28 days were used to calculate daily RGR.

Application of the Growth Model to Field and Experimental Data

The theoretical models for growth over the lifetime and during the nursery stage were applied to northern quahog growth data from Narragansett Bay and Point Judith Pond, Rhode Island. Weight-length data for northern quahogs in the size range of 60 to 140 mm were collected with a dredge in upper Narragansett Bay and analyzed for the a and b coefficients of the weight-length relationship. Data collected during the summer 1999 from an experimental-scale upweller in Point Judith Pond were used to define growth of the northern quahog during the first growing season.

TABLE 1.

Parameters used to model growth of the northern quahog in Rhode Island waters.

Coefficients		Samples Collected
L_∞	=	140 mm
t_o	=	0.10 years (36 days)
a	=	0.0004
b	=	2.80
K	=	0.1, 0.2, and 0.3

Specifically, the growth coefficient (K) was determined by non-linear regression methods (DeAlteris & Skrobe 2000); the time at length zero (t_o) was determined from a linear regression of length versus time after spawning; and the length-weight parameters (a and b) were calculated using linear regression of natural log (LN) transformed length and weight data.

RESULTS

Growth Over the Lifetime

The increase in shell length over the course of the northern quahog's 40-year life follows an asymptotic relationship (Fig. 1A). The shell length increases in an apparent linear pattern during the first two years of the northern quahog's life and then increases at a decreasing rate until the asymptote or maximum shell length is attained. The rate that the northern quahog reaches its maximum shell length varies substantially with varying K. The northern quahog reaches a maximum length of 140 mm in 11 years with a K of 0.30, in 17 years with a K of 0.20, and in 33 years with a K of 0.10. Unlike the growth function describing length over time, the weight (g) function resembles an apparent sigmoid-shaped relationship. The northern quahog's weight increases slowly during the first two years and reaches a maximum of 647 grams in 15, 23, and 39 years with a K of 0.30, 0.20, and 0.10, respectively (Fig. 1B). As the growth coefficient increases, the time required for the northern quahog to reach its maximum shell length and weight decreases. RGR over an annual time interval decreases from 3.3% increase/day during the first year to 0.5% increase/day during the juvenile years (age 1–2 years) and becomes negligible during the adult years (age 2+) (Fig. 2A). Although the same general trend was apparent for the SGR averaged over an annual time interval, the SGR during the juvenile years was about half the value of the RGR (Fig. 2B).

Growth During the First Growing Season

The increase in shell length during the first seven-month (210 day) growing season was linear, as would be expected from the von Bertalanffy growth curve (Fig. 3A). The maximum shell length reached during the first growing season varied considerably with K. The northern quahogs reached a shell length of 27 mm with a K of 0.30, a shell length of 18 mm with a K of 0.20, and a shell length of 9 mm with a K of 0.10. The increase in weight during the first growing season was exponential with a considerable lag during the first 40 days. Weights of 6.20, 2.20, and 0.30

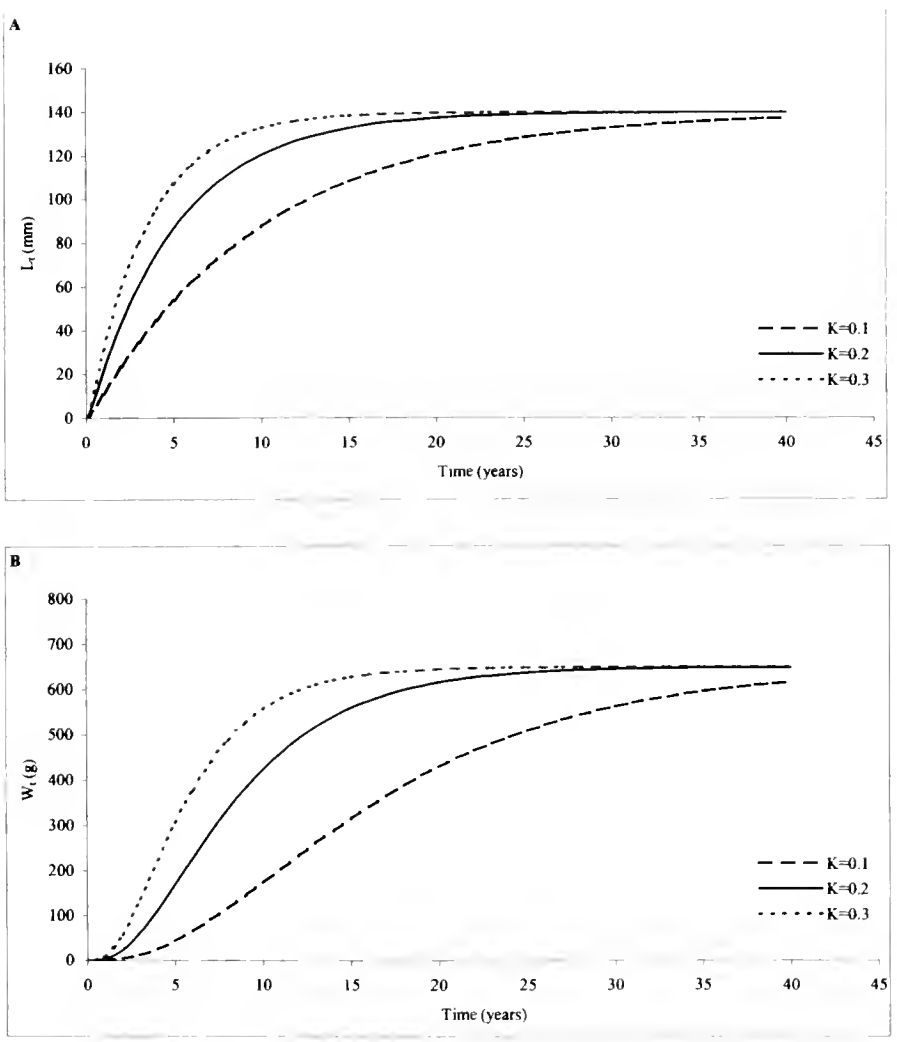

Figure 1. Length (A) and weight (B) at time for K values of 0.10, 0.20, and 0.30 over the life span of the northern quahog in Rhode Island waters.

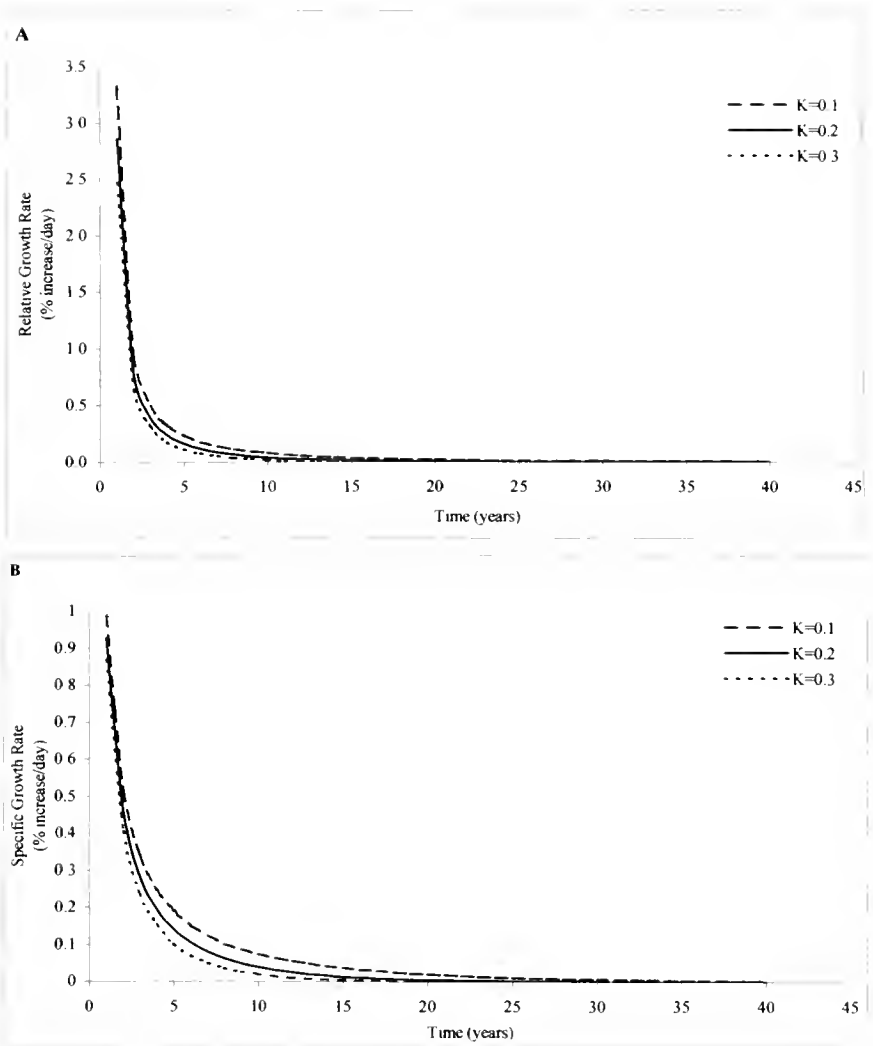

Figure 2. Daily relative growth rate (RGR) (A) and specific growth rate (SGR) (B) averaged on an annual basis at *K* values of 0.10, 0.20, and 0.30.

g were reached during the first growing season with *K* values of 0.3, 0.2, and 0.1, respectively (Fig. 3B).

The differences in final shell length and weight observed at *K* values of 0.10, 0.20, and 0.30 were not apparent when growth was converted to a RGR (Fig. 4A). Although there was a considerable change in the RGR during the first growing season, the northern quahogs displayed similar RGRs at varying *K* values. In particular, at 90 days after spawning, the northern quahogs grew at a rate of 11% increase/day, while at 150 days after spawning they grew at 3% increase/day. The RGR was high early in the northern quahogs first growing season, but decreased substantially and leveled off after 180 days post spawning. The SGR during the first growing season followed a similar pattern to the RGR (Fig. 4B).

The RGR was also determined using a variety of growth intervals. There were no detectable differences between RGR determinations at *K*s of 0.10, 0.20, and 0.30 with varying time intervals (Fig. 4A, Fig. 4B, Fig. 5A, Fig. 5B, Fig. 5C, and Fig. 5D); however, the value of RGR at a specific time varied with the time interval used to calculate RGR (Fig. 6). At 90 days after spawning, the northern quahogs grew at 26% increase/day based on a 28-day growth interval, at 15% increase/day based on a 14-day growth interval, at 13% increase/day based on a 7-day growth interval, at 11% increase/day based on a 4-day growth interval, and at 10% increase/day based on a 1-day growth interval. In other words, the larger the growth interval that RGR was averaged over, the larger the RGR. This relationship becomes less obvious when the northern quahogs reach 120 days after spawning and disappears after the first growing season (Fig. 6, Fig. 2A, and Fig. 2B).

Application of the Growth Model to Field and Experimental Data

For adult northern quahogs collected in upper Narragansett Bay, the linear regression of the LN of weight (g) versus the LN of length (cm) resulted in an R^2 of 0.94 (Fig. 7A). From this relationship, the *a* and *b* coefficients of the weight-length relationship were determined to be 0.00023 and 3.15, respectively.

For the nursery stage, the northern quahogs were spawned in 1999 April 8, at Bluepoints Company, Inc., West Sayville, New York. The linear regression of shell length versus the number of days after spawning was significant ($F(1, 6) = 132.87$, $p < 0.05$) with an R^2 of 0.96 (Fig. 8). From this relationship, t_o was estimated as 63 days after spawning. The linear regression of the LN of weight (g) versus the LN of length (cm) resulted in an R^2 of 0.99 (Fig. 7B). The *a* and *b* coefficients of the weight-length relationship were determined to be 0.00027 and 2.81, respectively.

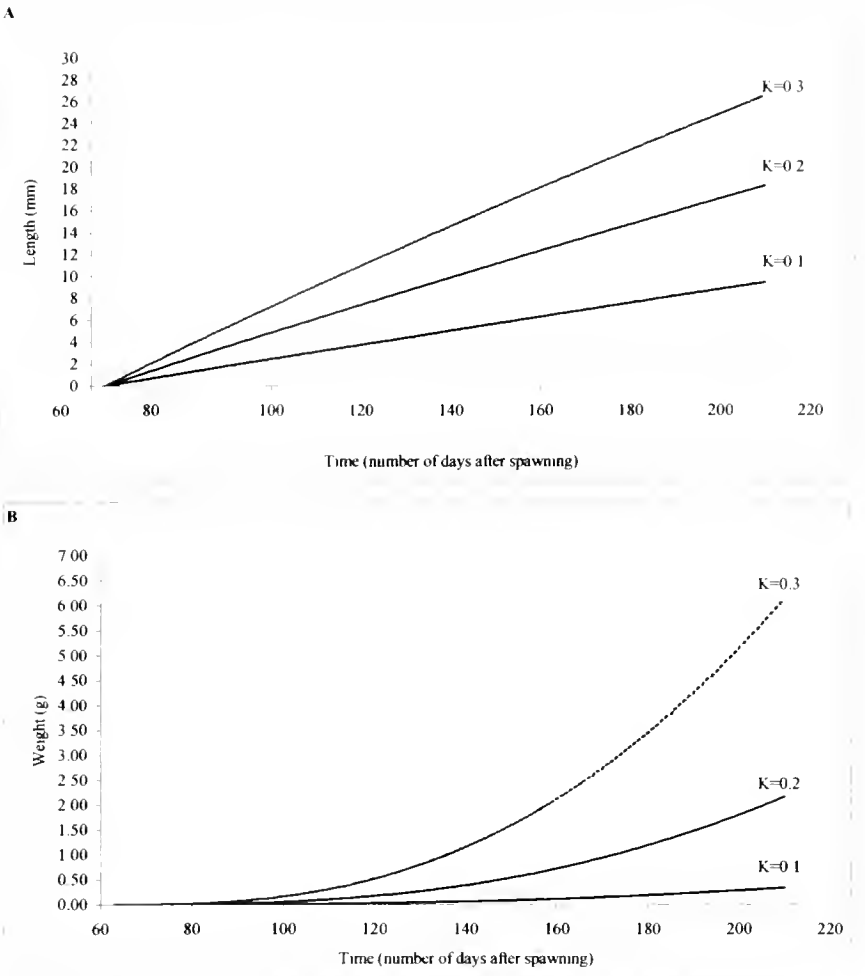

Figure 3. Length (A) and weight (B) at time for K values of 0.10, 0.20, and 0.30 during the northern quahogs first growing season.

A non-linear regression method estimated K to be 0.25 for the nursery stage growth experiment completed during the summer 1999 (Appleyard 2000) (Fig. 9). The predicted RGR was compared to the observed RGR based on a 4-day growth interval (Fig. 10). The experimental RGR varies considerably from the predicted RGR during the first 25 days of the experiment; the northern quahogs grew slower than expected. At approximately 100 days after spawning, the experimental RGR intersects the predicted curve; thereafter, the northern quahogs grew faster than expected, but tended to follow the general trend of decreasing RGR over time.

DISCUSSION

Growth Over the Lifetime

The results of this growth modeling exercise indicate that the growth rate of the northern quahog over its life span changes considerably (Fig. 1A and Fig. 1B) as a function of K. In particular, first year growth of the northern quahog is apparently linear for length and exponential for weight and both reach an asymptote as the animal ages. The age that growth reaches an asymptote depends on the particular growth coefficient (K) used. A higher K results in rapid growth and an earlier asymptote in the animal's maximum shell length and weight, while a lower K results in slower growth and a later asymptote; therefore, K is a useful indicator of environmental suitability for growth. Ansell (1968) reported the growth rates of adult northern quahogs throughout its geographical range based on annual growth rings. Within an acceptable temperature range, Ansell concluded that growth was determined by a variety of factors. Although the author successfully documented growth throughout the northern quahog's geographical range, he was unable to quantify why the northern quahogs were growing differently. A more useful measure would be the instantaneous annual growth coefficient (K). K incorporates the gamut of environmental conditions into one measure. Researchers could use K estimates to determine the health of the animal in a particular area and aquaculturists could utilize K estimates to determine the most suitable area for culture.

RGR averaged over an annual time interval decreases rapidly as the northern quahog ages, approaching 0% increase/day after age 2. RGR was relatively insensitive to changes in K. This has serious implications for experiments hoping to quantify differences in growth between treatments. Manzi et al. (1984) investigated northern quahogs in an experimental-scale upflow nursery system. The experimenters varied density to attain a range of chlorophyll-a effective flow rates and compared the growth, as expressed as RGR, between treatments. Although the investigators found qualitative differences in growth between treatments they

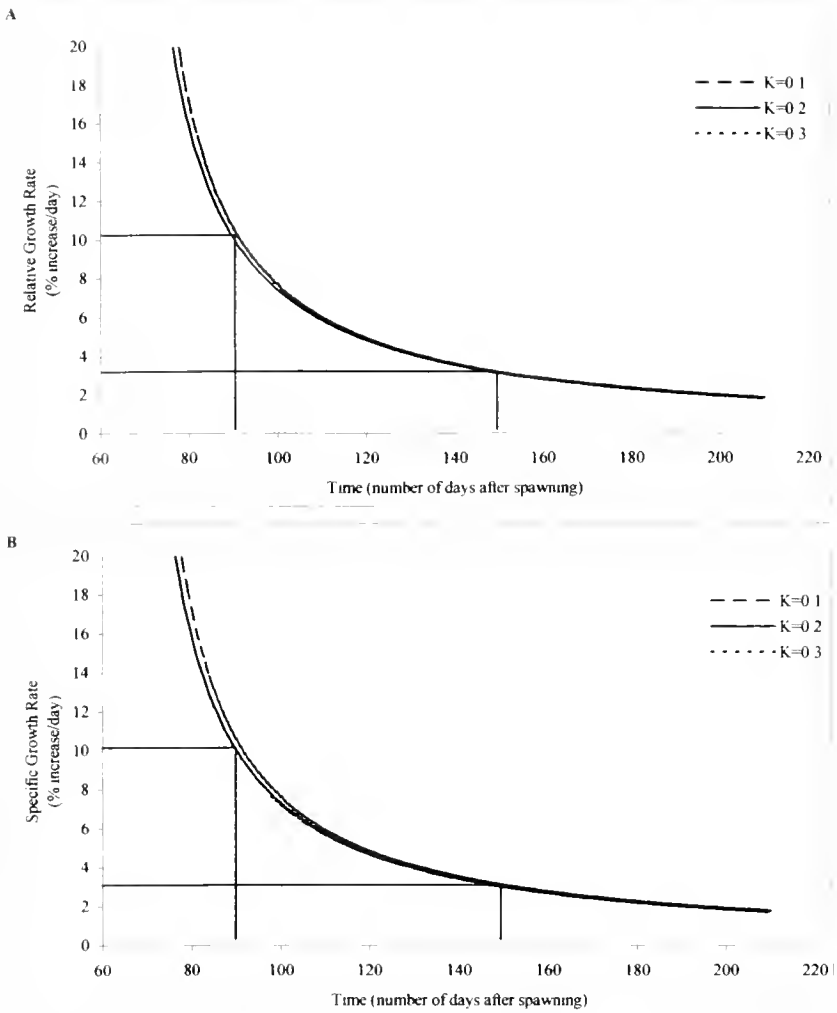

Figure 4. The relative growth rate (RGR) (A) and specific growth rate (SGR) (B) expressed as % increase/day at *K* values of 0.10, 0.20, and 0.30 during the northern quahogs first growing season in Rhode Island waters (calculated over a 1-day time interval).

did not find quantitative differences. Based on this modeling exercise, the authors would have been hard pressed to elucidate differences in growth between treatments based on RGR. A better measure to quantify differences between treatments would have been a change in length or change in weight.

SGR closely followed the patterns observed for RGR; however, because of the LN transformation, SGR during the juvenile years was about half the RGR. The LN transformation essentially compresses the growth scale and reduces the growth rate during the first two years. Comparisons between RGR and SGR are only appropriate for adult northern quahogs and should be avoided for juvenile animals. For example, Rheault and Rice (1996) measured SGR for oysters and scallops with an initial shell height of 45 and 43 mm, respectively. Their experiment monitored SGR during a six-week experiment. Since these bivalves are almost past the juvenile stage, SGR can be compared to RGR. To ensure an accurate comparison between this study and other similar experiments, SGR can be easily converted to RGR.

Growth During the First Growing Season

To predict growth during the nursery stage, growth of the northern quahog was investigated during the first growing season (210 days in Rhode Island). The increase in shell length and weight was linear. The final shell length and weight reached during the first growing season varied considerably with *K*; however, the RGR at different *K* values was almost identical. Again, the measure of RGR was relatively insensitive to changes in *K*. The RGR did vary during the first growing season. The RGR was extremely high early in the growing season (at 90 days after spawning the predicted RGR was 11% increase/day), but then decreased substantially and leveled off (at 150 days after spawning the predicted RGR was less than 4% increase/day).

The RGR was further investigated by altering the time period between RGR determinations. There were no observable differences between RGR at *K*s of 0.10, 0.20, and 0.30 with varying time intervals of 1, 4, 7, 14, and 28 days (Fig. 4, Fig. 5A, Fig. 5B, Fig. 5C, and Fig. 5D). In other words, RGR does not detect differences in the instantaneous growth rate (*K*). The value of RGR at a specific time varied with the time interval used to calculate RGR. Specifically, at 90 days after spawning there was a considerable difference between the RGR calculated over a 28-day interval and the RGR calculated over a 4-day interval (Fig. 6). Investigators should keep in mind that when comparing the results of growth experiments with different time intervals, observed differences in

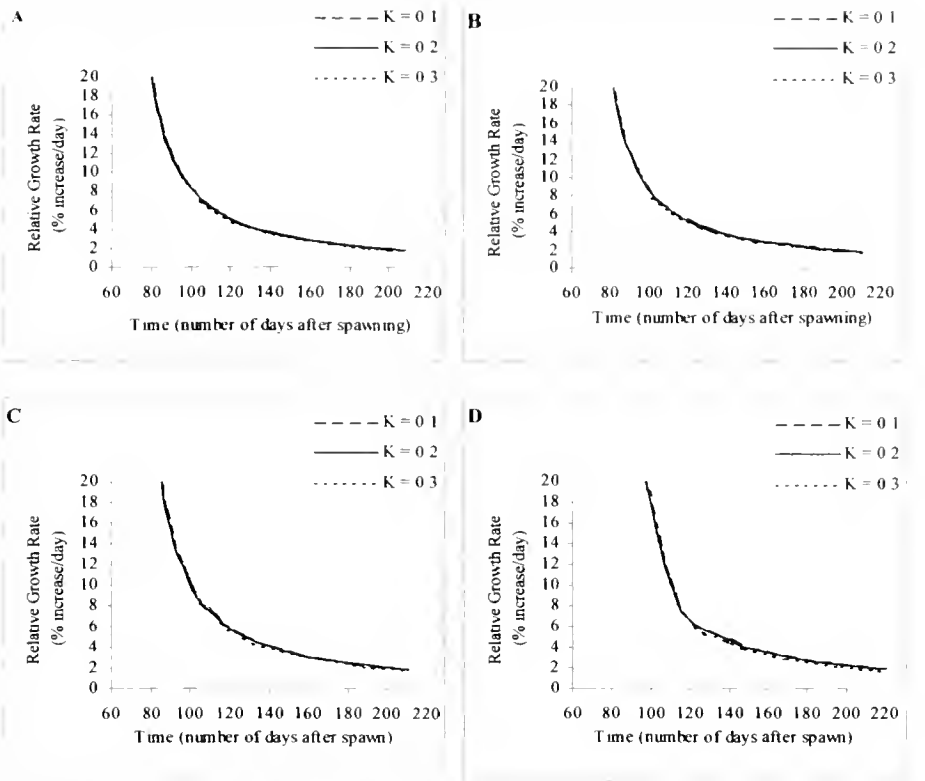

Figure 5. The relative growth rate (RGR) expressed as % increase/day over time at varying *K* calculated over: 4-day time interval (A); 7-day time interval (B); 14-day time interval (C); and, 28-day-time interval (D).

RGR may be due to differences in the time interval, not due to actual differences in growth.

Application of the Growth Model to Experimental Data

The *a* and *b* coefficients estimated for the weight-length relationship from the adults and nursery stage northern quahogs were different from each other and from other values published for Narragansett Bay northern quahogs. This suggests that researchers should always use data collected from locally available northern quahogs in a size range similar to that being modeled when estimating biomass from shell length and abundance data.

The relative growth rate changes considerably during the first growing season. The growth rate depends on the size of the animal as well as the time period between measurements. Growth studies on upwellers have not taken into account these differences. In addition, the variability between growth measurements has prohibited meaningful comparisons between research studies. Manzi et al. (1984) converted their biomass increase to a monthly percent increase because the time interval between volume determinations varied. The investigators failed to take into account changes in the growth rate at different time intervals. A study completed by Hadley et al. (1999) found a relationship between the daily growth rate (DGR) and flow ratio at varying northern quahog shell lengths. In particular, the authors found that the DGR increased as the size of the animal decreased. Based on the results of this

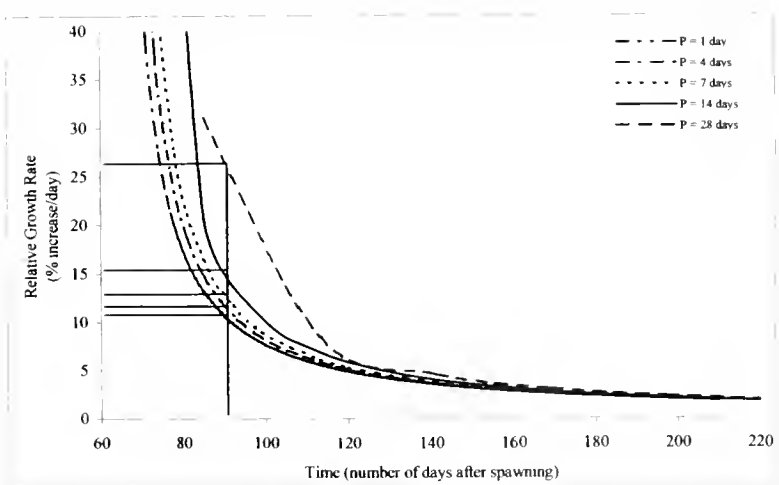

Figure 6. The relative growth rate (RGR) expressed as % increase/day over time at varying time intervals for a single *K* value of 0.20.

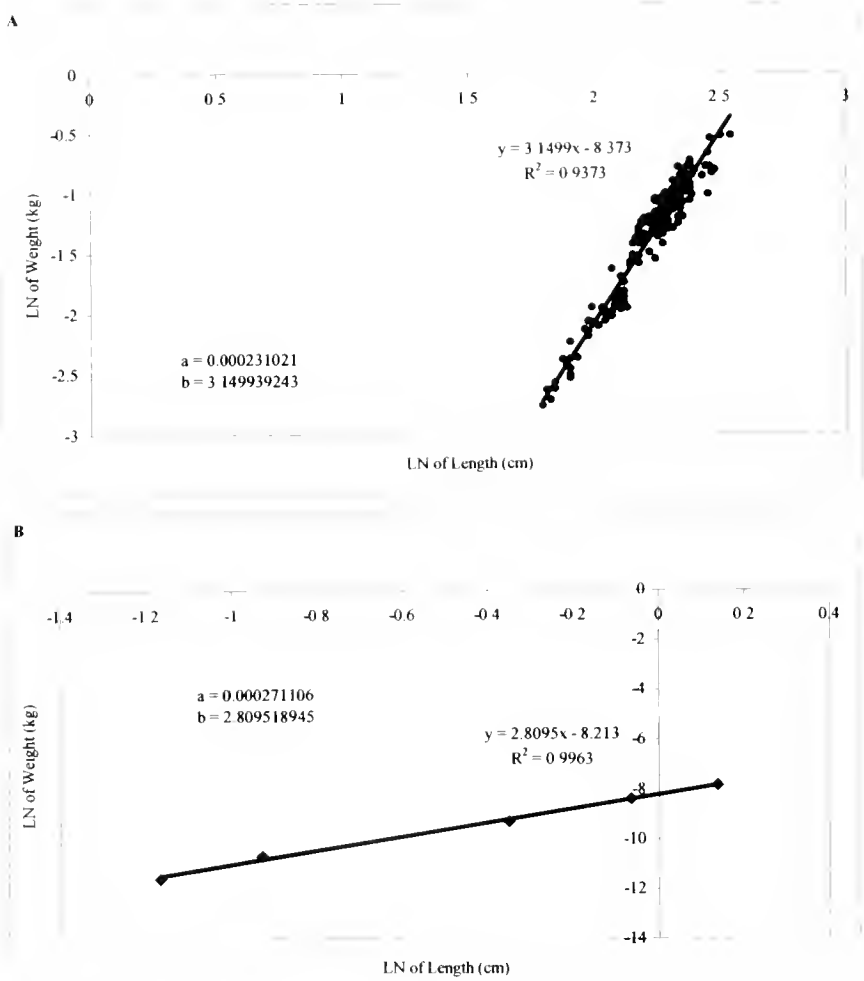

Figure 7. Linear regression of the natural log (LN) of weight (kg) and LN of length (cm) of adult northern quahogs sampled from Narragansett Bay (A) and of juvenile northern quahogs sample from an experiment in Point Judith Pond (B).

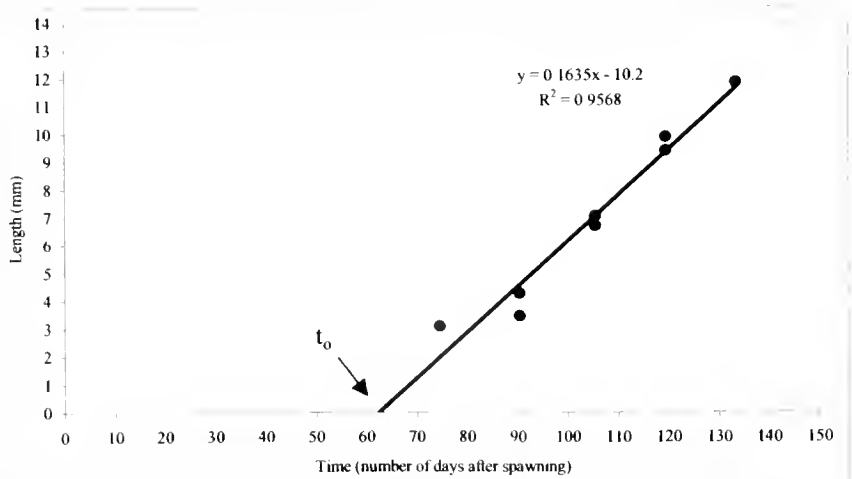

Figure 8. Linear regression of shell length (mm) with time (number of days after spawning) used to estimate t_o from juvenile northern quahogs sampled during the summer 1999.

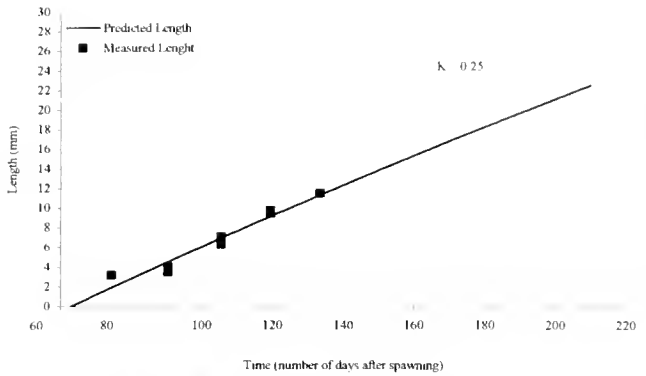

Figure 9. Observed shell length during the summer 1999 along with the predicted shell length at a *K* of 0.25 during the northern quahog's first growing season in Rhode Island waters.

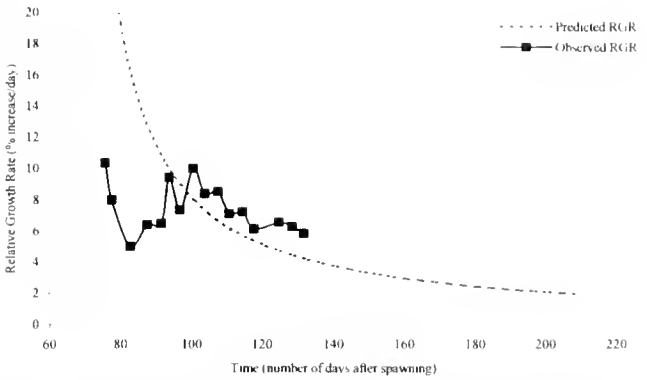

Figure 10. The predicted relative growth rate (RGR) versus the observed relative growth rate (RGR) during the first growing season on the northern quahog (calculated over a 4-day growth interval).

modeling exercise, the RGR is expected to increase with decreasing size. The relationship developed by the authors correlates well with predicted growth in the natural environment.

During the upweller experiment completed in the summer 1999 (Appleyard 2000, DeAlteris 2000), the northern quahogs grew with a *K* of 0.25, indicating average conditions for growth. A *K* of 0.25 approaches the maximum *K* observed by Jones et al. (1989) in Narragansett Bay, but in a culture situation, higher *K* values should be expected.

A predicted RGR was compared to the observed RGR during the aforementioned study. Early in the experiment, between 70 and 100 days after spawning, the experimental RGR differs markedly from the predicted RGR; however, 100 days after spawning the experimental RGR was higher than expected and followed the general trend of decreasing RGR over time. To incorporate the influence of anticipated growth on the experiment, the residuals between the predicted and experimental RGR were determined. The residuals were then compared to key environmental conditions to elucidate their effect on growth. Although there were no clear conclusions, this exercise was important in accounting for anticipated changes in growth over the first year of the northern quahog's life.

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REPRODUCTIVE STRATEGIES IN TROPICAL BIVALVES (*PTERIA COLYMBUS*, *PINCTADA IMBRICATA* AND *PINNA CARNEA*): TEMPORAL COUPLING OF GONAD PRODUCTION AND SPAT ABUNDANCE RELATED TO ENVIRONMENTAL VARIABILITY

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ABSTRACT The effects of environmental variability (El Niño 1997/1998) on three Caribbean bivalves (*Pteria colymbus*, *Pinctada imbricata* and *Pinna carnea*) were studied based on time series (1994 to 1998) of temperature, salinity, particulate organic matter (POM), larval and spat abundance. Monthly condition and gonad production cycles over a 12 months period are used to investigate whether these processes are coupled with recruitment.

The findings clearly show that El Niño 1997/1998 increased POM, decreased salinity and had little effect on temperature. In contrary to the Pacific, these changes in the study area were not caused directly, but by increased precipitation and no significant effects on larval or spat abundance were observed. The findings show similarities for the two closely related pearl oysters (*Pteria colymbus* and *Pinctada imbricata*), such as correlated larval and spat abundance cycles, and a negative correlation between temperatures and spat (whereas temperature was positively correlated with *Pinna carnea* spat). However, due to fewer spat peaks and a lower mean spat abundance, the uncoupling of gonad production and spat abundance, as well as less continuous gametogenic activity, a different reproductive strategy for *Pteria colymbus* is indicated.

KEY WORDS: bivalves, pearl oysters, fan shells, reproductive strategy, gonad production, larval abundance, spat abundance, El Niño, Caribbean

INTRODUCTION

Environmental variability can be seen in the context of latitudinal dependent environmental changes (i.e., seasonality) or interannual climate changes, (e.g., the climate anomaly El Niño). These environmental changes affect biological processes such as the reproduction and recruitment of marine species. Regarding seasonality it is generally accepted that as an adaptation to decreasing seasonality of environmental factors from polar to tropical regions (Lalli & Pearsons 1993) marine invertebrates from high latitudes have shorter spawning and recruitment periods, while at low latitudes longer and more continuous periods dominate. In the literature we find little published results on the relation between energy allocated to reproduction and temporal larval or spat variability in tropical benthic invertebrates. This might be explained by the fact that the reproductive output of animals with planktonic eggs and continuous reproduction are difficult to assess, and this reproductive strategy is likely to be very common among warm temperate and tropical benthic species (Crisp 1984).

El Niño might be regarded as an oceanographic phenomenon regionally restricted to the South American Pacific coast principally off Peru and North Chile which on statistical average occurs every three to seven years. Here, under El Niño conditions, the cold and nutrient rich upwelling system changes towards warm and nutrient-poor conditions that can lead to catastrophic consequences for the marine ecosystem (Arntz & Fahrbach 1991). Apart from effects on the marine ecosystem, impacts of El Niño on the terrestrial ecosystems of the South American continent, such as increasing rainfall in deserts and droughts in otherwise humid regions, can be observed. For example in the South American Caribbean, which is not affected directly by El Niño, different precipitation patterns lead to secondary (indirect) oceanographic changes, and thus effects upon the marine ecosystem can be assumed. Among the many reports of El Niño impacts on marine ecosystems, the results of Urban (1994) and Urban and Tarazona (1996) are most relevant to this study. They postulate that for several bivalve species, El Niño normally only affects the energy balance (such as metabolism, growth rate, gonad output), larval survival and recruitment. These two studies so far seem to be the only long-term approaches quantifying the effects of El Niño on marine benthic invertebrates of South America.

Pearl oysters (family Pteriidae) and fan shells (family Pinnidae) are of commercial importance, as reflected by the quantity of publications devoted to aquaculture and spat settlement (for pearl oysters see review in Urban 2000a). For fan shells (different *Atrina* species and *Pinna rugosa*), see studies on ecology, fishery and aquaculture by Arizpe and Félix (1986), Cendejas et al. (1985), Baqueiro and Castagna (1988), Davy and Graham (1982), Philipson (1989), Turner and Rosewater (1958). In the Caribbean waters of Colombia, the dominant pearl oysters are *Pteria colymbus* and *Pinctada imbricata* and the fan shells are represented by *Atrina serrata*, *Atrina seminuda* and *Pinna carnea*, with the latter being the most dominant. *Pteria colymbus* lives attached to octocoral communities, *Pinctada imbricata* was found to be the dominant sessile species in sea grass (*Thalassia testudinum*) communities (Urban 2000a) and *Pinna carnea* favors medium to coarse sandy substrates or mixed substrata (sand-rocks-corals). All three species are epifaunal species distributed in depths between 0.5 m to 15 m. Except for Urban 2000a and Urban 2000b on the aquaculture and population dynamics of *Pinctada imbricata*, no further information on pearl oysters and fan shells from this region was found in the reviewed literature.

The objectives of this paper are to study possible effects of oceanographic changes caused by El Niño on reproduction and recruitment of three tropical bivalve species. Furthermore, the cyclic reproductive patterns and temporal coupling of gonad production and spat variability are investigated. This includes duration of larval development under laboratory conditions and spat growth experiments. Reproductive strategies are discussed.

MATERIALS AND METHODS

Study Area

Spat and larval abundance and environmental factors, were monitored monthly between 1994 and 1998 in Chengue Bay, Tay-

rona National Nature Park (11°20′N, 74°10′W, Fig. 1a). Gametogenic activity and condition cycles were studied monthly during one year, but during different years for each species: *Pteria colymbus* (1,354 specimens) were sampled between March 1994 and February 1995 in Tayrona Park, *Pinna carnea* between April 1995 and April 1996 (1469 specimens from Tayrona Park) and *Pinctada imbricata* between March 1997 and March 1998 (782 specimens) in the "Cabo de la Vela", a Peninsula located in the Guajira province (12°10′N,72°20′W and 12°00′N, 72°10′W), approx. 300 km from the Tayrona Park (for detailed description of the Cabo de la Vela study area see Urban 2000a, Fig. 1b). Tayrona Park consists of several small bays offering a great variety of substrates (patches of sea grass, coral reefs, sandy-silty substrates and mixtures of coral and sand bordered by mangroves). Annual water temperatures vary between 26°C and 30°C with a mean salinity of 36‰ and a tidal range of less than 30 cm.

Sampling

Monthly zooplankton samples were taken between 9:00 and 11:00 a.m. with a Bongo net (○ 35 cm, mesh size 150 µm, filtering 25–30 m^3 seawater). Samples were preserved in 70% alcohol and all bivalve larvae of the genus *Pinctada*, *Pteria* or of the family Pinnidae were identified and counted. For larval identification, "morphotypes" from laboratory reared larvae, were compared with larvae obtained from zooplankton samples. Spat abundance was determined deploying collectors (="traps") that consisted of three plastic mesh "onion bags" (80 × 30 cm, mesh size 0.8 cm), protected by a propylene net-bag. Collectors were tied to a bottom long line at 5 and 10-m depths, and recovered 6 weeks later. Mean abundance collector^{-1} was determined at monthly intervals. At each sampling date bottom temperature and salinity were recorded and seston samples were taken at 3-m water depth with a pump. Seston samples were filtered using Whatman Glass Microfibre filters, which were dried for 24 hours at 70°C. Particulate organic matter (POM) was calculated after ignition of filters at 500°C for 4 hours.

Gametogenic Activity, Condition and Gonad Production

Maximum length (anterior-posterior axis) was recorded for all specimens with vernier calipers. Two sub-samples of 30–40 individuals each were selected randomly covering the entire size range. One sub-sample was used for gametogenic analysis. Every month the reproductive stage of 30 individuals was determined based on microscopic observation on fresh gonad material (smear samples) using a semi quantitative scale (Urban 2000a: indifferent, developing 1, developing 2, ripe, spent). With the second subsample the condition (body weight cycle) was determined. Soft parts were removed and dried at 70°C to constant weight to determine shell free dry weight (SFDW). Ash free dry weight (AFDW) was obtained by ignition of dried soft parts at 550°C for five hours. Parameters "a" and "b" of the monthly relationships between shell length and shell-free dry weight (Eq. 1) were estimated with non-linear regression analysis. An annual shell free dry weight (i.e., condition) cycle for a standard individual was calculated as

$$SFDW = a \cdot SL^b \quad (1)$$

where SFDW is the shell free dry weight [g] and SL the shell length [mm]. Gonad production was estimated using a new method (for details see Urban & Riascos submitted) based on monthly quantitative samples, monthly length-gonad weight relationships, pooled annual length frequency data and observation of the gametogenic activity. First the gonad region was replaced by geometric abstractions. With linear measurements of the gonad region and geometric equations corresponding to geometric bodies, the gonad volume and thus the monthly gonad weights were estimated. Assuming the gametogenic stage "ripe" corresponds to the part of the population which spawns during a particular month, the monthly spawned gonad weight for each size interval was estimated. Finally, the annual gonad production of the entire population is given by the sum of all months and length class intervals according to Eq. 2.

$$W_{gon\_ann\_pop} = \sum [W_{gon_{i,j}} \cdot P_{spawn_{pop_{i,j}}}] = \sum \left[\left(\frac{V_{gon_{i,j}}}{V_{tot_{i,j}}} \cdot W_{tot_{i,j}} \right) \cdot \left(\frac{\left(\frac{n_i}{\sum n} \cdot A \right) \cdot ripe_j}{100} \right) \right] \quad (2)$$

$W_{gon\_ann\_pop}$ annual gonad production of the population [g AFDW m^{-2} yr^{-1}]
$W_{gon\,i,j}$ gonad weight per length interval "i" during month "j" [g AFDW]
$P_{spawn_{pop_{i,j}}}$ part of the population spawning per length interval "i" during month "j"
$V_{gon\,i,j}$ gonad volume per length interval "i" during month "j" [ml]
$V_{tot\,i,j}$ total body volume per length interval "i" during month "j" [ml]
$W_{tot\,i,j}$ total body weight per length interval "i" during month "j" [g AFDW]
n_i number of specimen in length interval "i"
Σn total number of specimen of the pooled length frequency sample [yr^{-1}]
A mean abundance from quantitative samples [n m^{-2}]
$ripe_j$ part of the population with gametogenic stages "ripe" during month j [%].

Gonad production could not be estimated for *Pinna carnea* due to lack of quantitative samples.

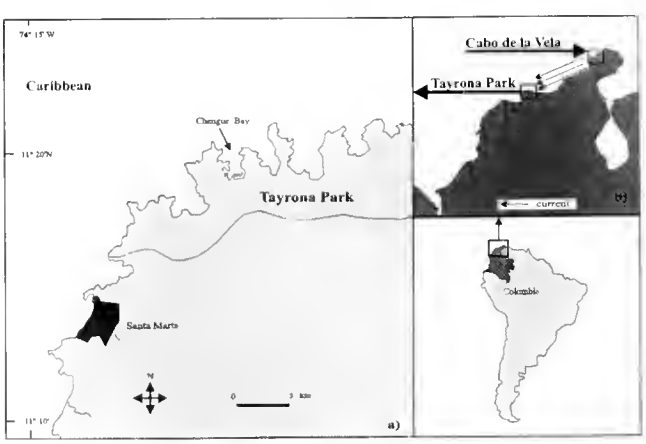

Figure 1. Study area. Tayrona Park, Caribbean of Colombia.

Larval Development and Settlement

Adults of *Pinctada imbricata* were adapted for four weeks to laboratory conditions (ultraviolet-irradiated, 45 µm filtered seawater, 23°C–25°C, 34‰, fed on a 1:1 mixture of *Chaetoceros gra-*

cilis and *Isochrysis galvana*). During a spawning trial the temperature was gradually increased every 10 minutes up to 34 C. Fertilization was initiated mixing eggs and sperm in a 1:10 ratio. Larvae (three replicates) were transferred to 500 L tanks at an initial density of 6 larvae ml^{-1} and fed on different diets of *Isochrysis galvana* and *Chaetoceros gracilis* (to day 15: 50.000 cel. ml^{-1}, after day 15: 100.000 cel. ml^{-1}). Water was changed every two days. From day 15 on, a propylene net material was placed in the tanks during larval settlement. Larval growth was studied for 37 days by microscopic measurement of the anterior-posterior length of approximately 10–15 larvae every 5 days. From day 47 to day 88 the growth rate of 50 post-larvae (initial shell length 2.9 mm ±0.675 S.D.) placed in pearl nets (three replicates) under natural conditions (Urban 2000b) was followed by measuring 10–15 individuals from each replicate every 5 days.

Analysis of Time-Series Data

In order to study possible cyclic patterns of larval and spat abundance, as well as abiotic data (temperature, salinity and organic matter), time series were analyzed with autocorrelation plots and Fourier analysis. Data were smoothed with moving averages (over four), interpolating missing data. Furthermore, time series were normalized according to "(x-mean)/standard deviation" and the positive values surpassing the mean, i.e., the positive peaks were plotted.

RESULTS

Larval and spat growth can be described by power functions yielding high determination coefficients (Fig. 2). Larvae took 17 days to grow from straight-hinged larvae to pediveliger (8 μm day^{-1}). Thereafter, during the following 11 days from pediveliger to spat it was 83 μm day^{-1}, and finally, during the last 10 days of this particular experiment, growth increased to 112 μm day^{-1}. From day 47, mean growth of spat under natural conditions was 198 μm day^{-1}.

Figure 3 shows the gametogenic activity and condition cycles, recorded during three different years (Fig. 3a), the larval and spat abundance (Fig. 3b, 3c, 3d), and the environmental factors (Fig. 3e, 3f) for the entire period (1994–1998), in order to give a general overview of the available data. Autocorrelation plots (not shown) on all time series data (i.e., data of Fig. 3b, 3c 3d, 3e, 3f), except for temperature, were highest and significant (surpassing the 95% confident interval) at Lag 1 (with rather low values between 0.484 and 0.622) or at Lag 2 (with values between −0.604 to −0.725). In

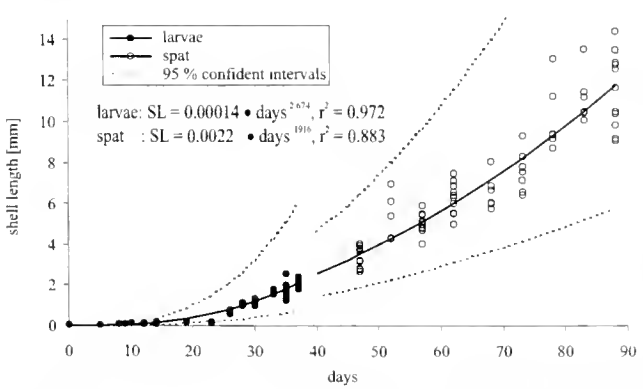

Figure 2. Larval growth under laboratory conditions and spat growth under natural conditions of *Pinctada imbricata*.

time series data we expect significant correlation at low Lag's (i.e. a particular value depends on a value one time unit earlier). However, the lack of significant correlation at higher Lag's, indicates the absence of a cyclic pattern of these variables, which was confirmed by Fourier analysis. Significant values were only obtained for temperature at Lag 1 (=0.536), Lag 6 (=0.763) and Lag 12 (=0.480), indicating a 12-month cycle, which was confirmed by Fourier analysis giving one high peak (of square magnitude) at a period of 10.7 months.

The El Niño effects on oceanographic factors show less temperature and salinity and more POM peaks from 1997 on (= beginning of El Niño, Fig. 4). This indicates a strong salinity and a slight temperature decrease as well as a strong POM increase. In order to correct the El Niño effects which mask the salinity and POM peaks, these two factors were normalized for two periods (pre-El Niño: March 1994 to December 1996, El Niño: January 1997 to August 1998). To quantify the relation between all time series, a Spearman correlation analysis was carried out (Table 1). Larval cycles could not be related conclusively to spat cycles (Fig. 5). For example in *Pteria colymbus* the first two larval peaks occurred immediately after the spat peaks. The Spearman correlation confirmed these results giving low values of <=0.2 between larval and spat cycles of the same species. However, the larval cycles of all three species seem to follow a similar pattern, given by higher correlation values (*Pteria colymbus/Pinctada imbricata* = 0.6, *Pinna carnea/Pinctada imbricata* = 0.7 and *Pteria colymbus/Pinna carnea* = 0.4). Low values were found for the correlation between any of the environmental factors and larval abundance and a negative correlation between temperature and salinity was indicated (−0.5). During the entire study period (54 months), *Pteria colymbus* exhibited only four annual short peaks between December to March of each year, whereas *Pinctada imbricata* and *Pinna carnea* had seven peaks in the same time (Fig. 5, Fig. 6).

The normalized cycles of SFDW (a), gonad production (b), spat abundance (c) and temperature as well as salinity (d) are given in Figure 7. Due to the fact that gonad production could not be estimated for *Pinna carnea* (see methods), in this case the gametogenic stage "ripe" is shown (Fig. 7b). For *Pinctada imbricata* SFDW, gonad production and spat abundance cycles agree extremely well as in all cycles 3 peaks are found in the same months (beginning, middle and end of the year, indicated by black arrows). For *Pinna carnea* SFDW and "ripe" are not well coordinated; SFDW peaks in the second half of the year and the reverse is true for the "ripe" cycle (with 2 peaks in the first half of the year), however "ripe" and spat peaks are in line. For *Pteria colymbus* SFDW and gonad production have 2 peaks in the first half of the year while the spat cycle with one peak at the end of the year does not seem to be related at all. No conclusive relationship is indicated with either of the environmental factors. In 1994 and 1995 temperature and salinity cycles peak at different times of the year, indicating a negative correlation, while in 1997, with the beginning of El Niño, this pattern seems to have changed as salinity and temperature peak at the same time.

Finally, the effects of El Niño on environmental factors and spat variability are summarized (Fig. 8). Clearly the El Niño had an effect on salinity and POM, as salinity on average decreased from 36.25‰ to 34.75‰ and POM increased from 0.25 to 0.6 mg l^{-1}. However, no conclusive results were obtained indicating that these oceanographic changes had any significant influence on spat abundance, though *Pteria colymbus* seems to typically have a lower spat abundance.

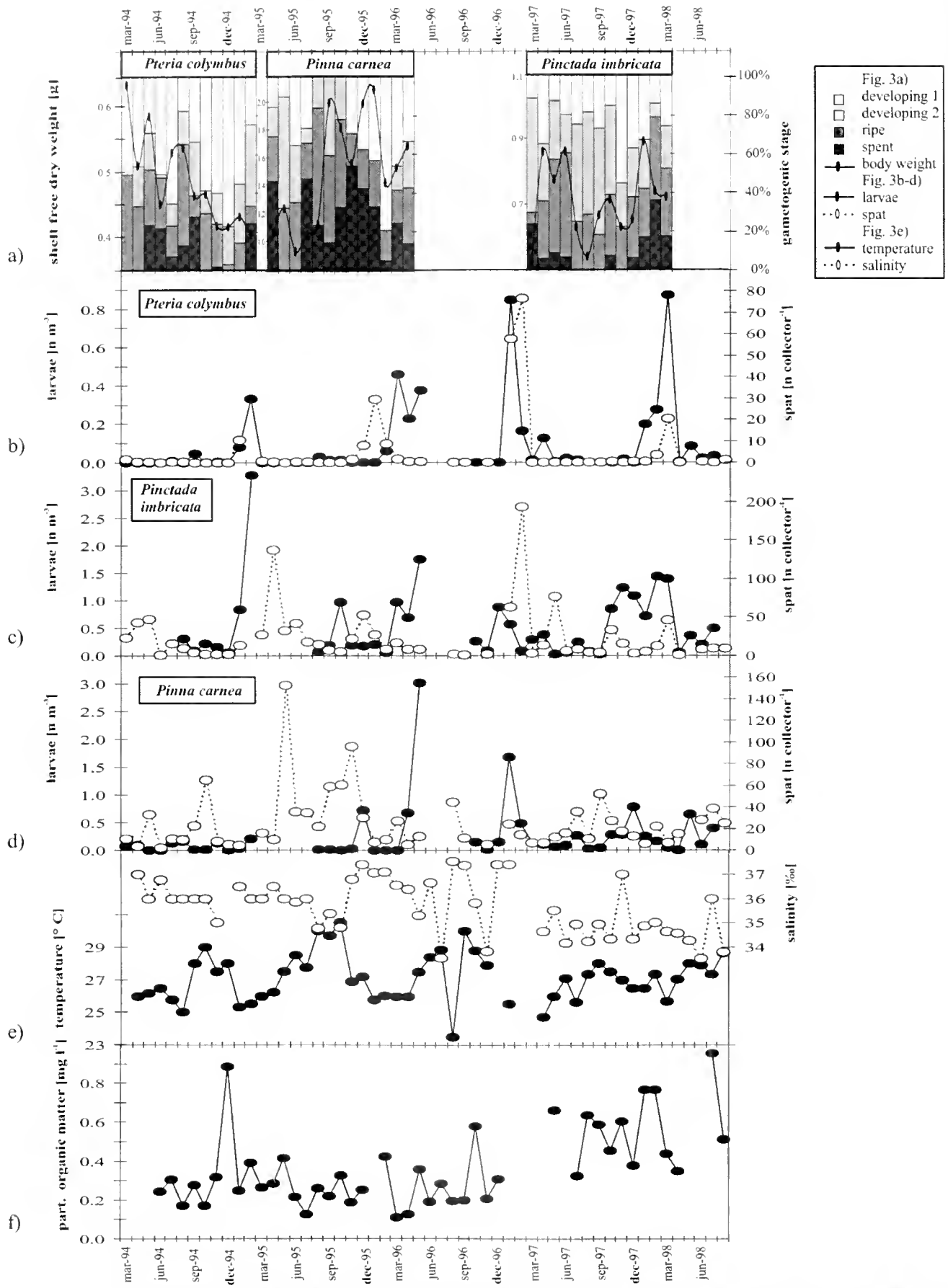

Figure 3. Reproductive cycles and time series. a) One year cycles of gametogenic activity and shell free dry weight cycle (*Pteria colymbus*, *Pinna carnea* and *Pinctada imbricata*), b-d) larval and spat abundance between March 1994 and August 1998 (b: *Pteria colymbus*, c: *Pinctada imbricata*, d: *Pinna carnea*), e) temperature and salinity, f) particulate organic matter.

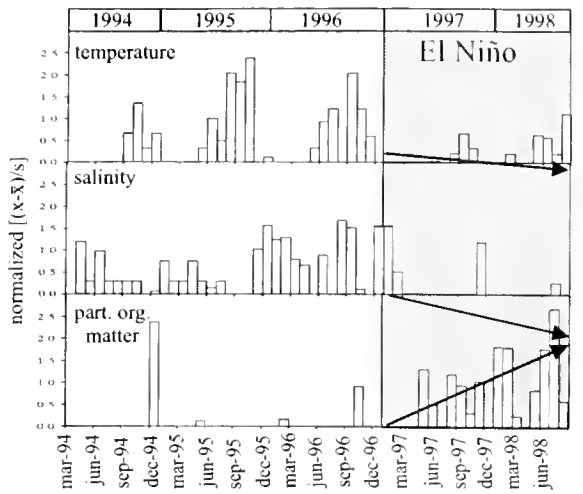

Figure 4. El Niño effects on temperature, salinity and particulate organic matter. Decreasing or increasing tendencies are indicated by arrows.

DISCUSSION

In areas with a strong seasonality, reproduction (maturation and spawning) is often found to be triggered directly by temperature (Alagarswami 1966, Urban & Campos 1994). Alternatively, many reports on temperature as an indirect trigger have been published. For example larvae abundance (*Mytilus edulis*, and *Macoma balthica* larvae from the North Sea) are correlated to algal bloom in summer that depends on temperature (Niesel & Günther 1999). In the current study under tropical conditions we would have expected that reproduction depends on salinity and food availability rather than on temperature (Barnes 1957) because compared to temperate and Polar Regions in the tropics, annual temperatures are less variable. However, this prediction could not be confirmed, on the contrary spat abundance was best correlated with temperature.

Recruitment (i.e., spat abundance) that follows after reproductive processes such as gonad production and spawning can be assumed to be temporally coupled. However, the literature gives numerous examples where reproduction and recruitment are highly variable and uncoupled. This is explained by factors such as patchy food supply for larvae (Langdon 1983), thus, causing an extension of the developmental period and a reduction of survival owing to larval food limitation (Olson & Olson 1989). Furthermore, oceanographic processes influencing larval dispersal (Strathman 1974) and—especially in tropical regions—longer continuous reproductive periods uncouple reproductive processes. This study confirmed these results in the case of larval and spat abundance: Although in several cases larval and spat abundance is in line, the general picture is that both cycles are variable following different patterns. Apart from the possible explanations stated above, this could be due to the fact that zooplankton samples were taken only monthly. Thus, peaks could easily have been missed, whereas spat abundance that was recorded from collectors left for six weeks could be assumed to be more reliable. This aspect is important because at least for *Pinna carnea* and *Pinctada imbricata*, reproduction (gametogenic activity or gonad production) correlate extremely well with spat abundance while in *Pteria colymbus* these processes seem to be uncoupled. Owing to a lack of data for *Pinna carnea*, instead of the gonad production the gametogenic stage "ripe" is given. Urban and Riascos (submitted) found the monthly gonad volume cycle and the gametogenic stage "ripe" cycle of *Pinna carnea* from Tayrona Park to be positively correlated, explaining this finding as follows: The gametogenic stage "ripe" gives the percentage of specimens with ripe gonads (shortly before spawning). Gonads are most voluminous in this development stage as egg and sperm size is large. As the method to estimate gonad production is based upon the monthly gonad volume, gametogenic stage "ripe" and gonad production are also correlated.

The apparent "direct" coupling of reproduction and recruitment in *Pinna carnea* and *Pinctada imbricata* at first appears suspicious because a time lag would have been expected, i.e., spat peaks and gonad production peaks should not be observed at the same time. Larval periods of bivalves have been the subject of numerous studies, and it is obvious that their duration depend principally on temperature and food. For example, the larval development of *Donax serra* from the productive and temperate Benguela upwelling system lasts 3–4 weeks (Birkett & Cook 1987, Donn 1987), and for the tropical *Pinctada maxima* the larval development time is 28–35 days (Rose & Baker 1994). Besides this "time problem" it has to be taken into account that gonad production of *Pinctada imbricata* was recorded for a population in Cabo de la Vela, 300 Km from Tayrona Park (where spat experiments were carried out). However, *Pinctada imbricata* spat from collectors after 6 weeks ranged from 1–4 mm shell length, which is in good agreement with larval growth experiments in which spat after 6 weeks had a mean length of 2.8 mm. Furthermore, laboratory-reared spat used for growth experiments after 6.7 weeks measured 2.9 mm (±0.675 S.D.). The question remains whether larvae could have been transported from Cabo de la Vela to Tayrona Park within this time. The Caribbean Current (Thurman 1994) flows parallel to the Colombian coast into the Gulf of Mexico with a velocity of 0.5 m s^{-1} (Tomczak & Godfrey 1994), thus spawned *Pinctada imbricata* larvae can be transported from the Cabo de la Vela to Tayrona Park within 6.9 days (Fig. 1b). According to laboratory experiments, larval development until settlement lasts 23–28 days. Assuming a similar development time for *Pinna carnea*, "collector periods" of six weeks would lead to such overlapping peaks of recruitment and reproduction as found in Figure 6, with no time lag between coupled gonad production and spat peaks being observed.

At high latitudes seasonality is principally governed by the large annual temperature variability, and at low latitudes (i.e., the tropics) with more constant temperatures, there is little seasonality. However, a clear seasonality of environmental factors other than temperature can be observed in tropical regions. In the study area a combination of precipitation and wind patterns seem to be the principal driving force. Annually, the rainy season (normally between June and November) is followed by the dry season (normally between December and May). Precipitation decreases salinity and the run off of nutrients increases POM. On the other hand a local upwelling (Blanco 1988) towards the end of the rainy season leads to a drop of mean temperature (?4°C). This explains the apparent negative correlation between temperature and salinity.

As mentioned above, El Niño increases precipitation in the semi-arid region of the Colombian Caribbean close to Venezuela. Thus, decreasing salinity, increasing POM and negligible effect on temperature during El Niño can be postulated. This is an interesting parallel comparing El Niño effects in the South American Pacific where temperature increases, and (in the pelagic regions), nutrients decrease. In the Caribbean, salinity variability owing to

TABLE 1.

Spearman correlation matrix of larval, spat, temperature, salinity and particulate organic matter (POM) cycles from March 1994 to August 1998. Values > = 0.5 are printed bold

	Larvae			Spat				
	Pteria colymbus	*Pinctada imbricata*	*Pinna carnea*	*Pteria colymbus*	*Pinctada imbricata*	*Pinna carnea*	Temperature	Salinity
P. imbricata	**0.6**							
Larvae *P. carnea*	0.4	**0.7**						
P. colymbus	0.1	0.4	0.1					
Spat *P. imbricata*	0.1	0.2	0.1	**0.6**				
P. carnea	−0.2	−0.1	−0.1	−0.2	0.1			
Temperature	−0.2	−0.3	−0.2	**−0.5**	−0.4	0.4		
Salinity	−0.1	0.0	0.0	0.2	0.3	0.0	**−0.5**	
POM	0.2	0.1	0.1	0.0	0.0	−0.2	0.1	0.0

El Niño most likely has little influence on the ecosystem. In the Pacific however, the reduction of nutrients during El Niño, leads to a characteristic breakdown of food chains (Glynn 1988, Enfield 1988, Arntz & Fahrbach 1991). Considering that in the Caribbean during El Niño 1997–1998 POM increased almost three fold, it could be assumed that suspension feeding secondary producers such as bivalves benefited from the increased POM pool, gaining more energy for production.

However, except for *Pinctada imbricata*, no differences in larval or spat abundance that could be attributed to El Niño-induced changes of environmental factors were observed in this study. Moreover, *Pinctada imbricata* rather showed a reduction of spat abundance during El Niño. This finding might be explained by noting that filtration rates of filter feeding bivalves are positively

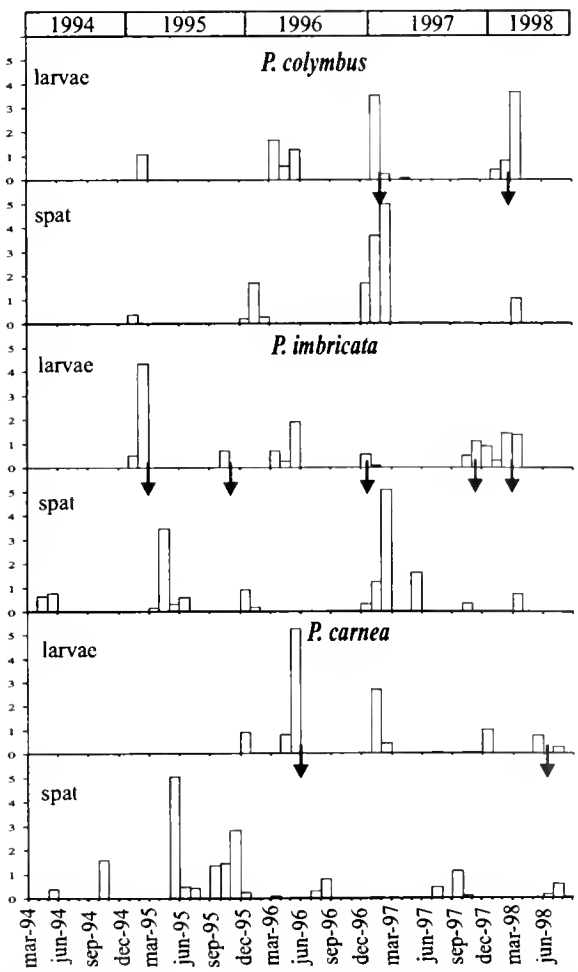

Figure 5. Larval and spat abundance of *Pteria colymbus*, *Pinctada imbricata* and *Pinna carnea*. (Black arrows indicate a good agreement between larvae and spat peaks, i.e., a larvae peak shortly before a spat peak).

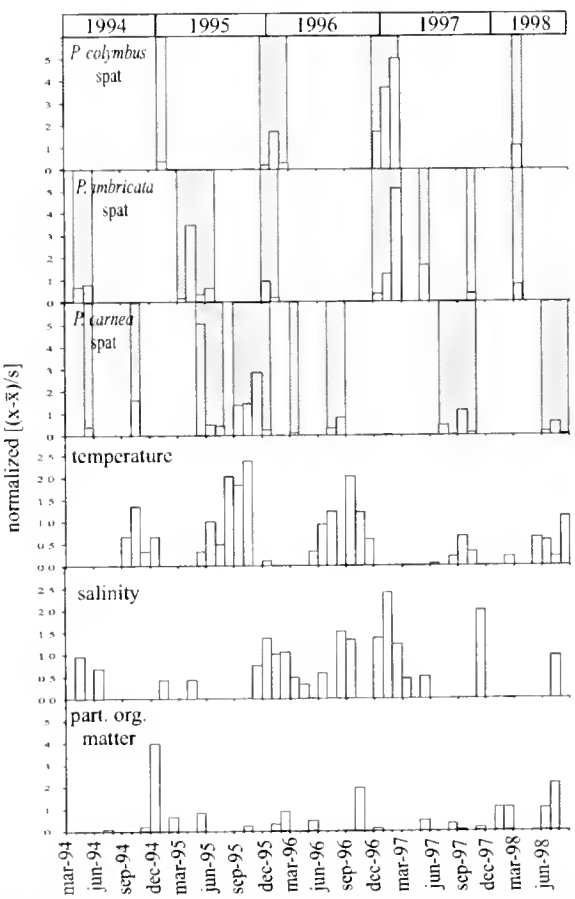

Figure 6. Spat abundance of *Pteria colymbus*, *Pinctada imbricata* and *Pinna carnea* compared with temperature, salinity and particulate organic matter. (Periods of spat peaks are indicated with gray bars).

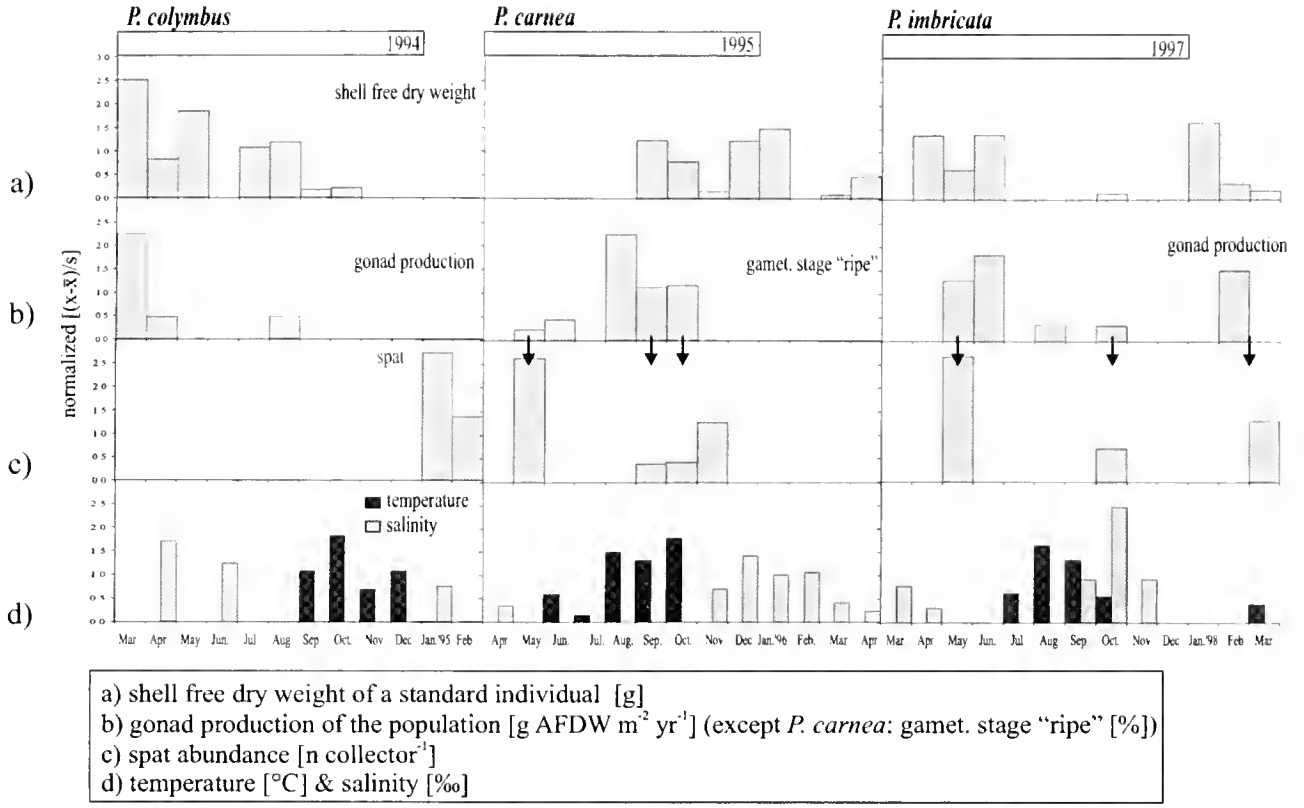

Figure 7. Temporal coupling (black arrows) of gonad production and spat abundance of *Pteria colymbus* (March 1994 to February 1995) *Pinctada imbricata* (April 1995 to April 1996) and *Pinna carnea* (March 1997 to March 1998).

related to particle concentration only up to a certain optimum concentration (Jørgensen 1960, Foster-Smith 1975). Should the POM concentration surpass this optimum no extra surplus energy would be assimilated.

As to discuss reproductive strategies we have three epifaunal filter feeders distributed in the same study area. The principal differences in their niches seem to be substrate preference (*Pteria colymbus*: octocorals, *Pinctada imbricata*: sea grass community, *Pinna carnea*: buried semi-infaunal). They all display continuous reproductive cycles, typical for tropical regions with spent gonads during at least seven months of the year, and larvae as well as spat throughout most of the year. In contrast, for the clam *Gari solida* from Southern Chile (Urban & Campos 1994) and for the Antarctic bivalve *Laternula elliptica* (Urban & Mercuri 1998) more discontinuous reproductive cycles were observed with principally spent gonads present only during 2–3 months of the year.

Apart from common characteristics, this study revealed the following differences: The two closely related pearl oysters (*Pteria colymbus* and *Pinctada imbricata*) show several similarities such as high correlation between larval as well as spat cycles. In comparison, spat cycles of *Pinna carnea*, are not correlated with either of the spat cycles of both oysters. Furthermore, for both pearl oysters temperature is negatively correlated with spat, (and positively correlated with *Pinna carnea* spat). On the other hand, *Pteria colymbus* had only half of the spat peaks, and thus less mean spat abundance along with uncoupling of gonad production and spat cycles that clearly displays differences from *Pinctada imbricata*. Also, gametogenic activity of *Pteria colymbus* is less continuous than the latter two species, because at least during a two-month period (December 1994 to January 1995) little or no spent or ripe gonads were present. Thus, on a scale from discontinuous reproduction of polar species to continuous reproduction of tropical species as the extremes, we would find *Pteria colymbus* in a more intermediate position inclined towards the "tropical group".

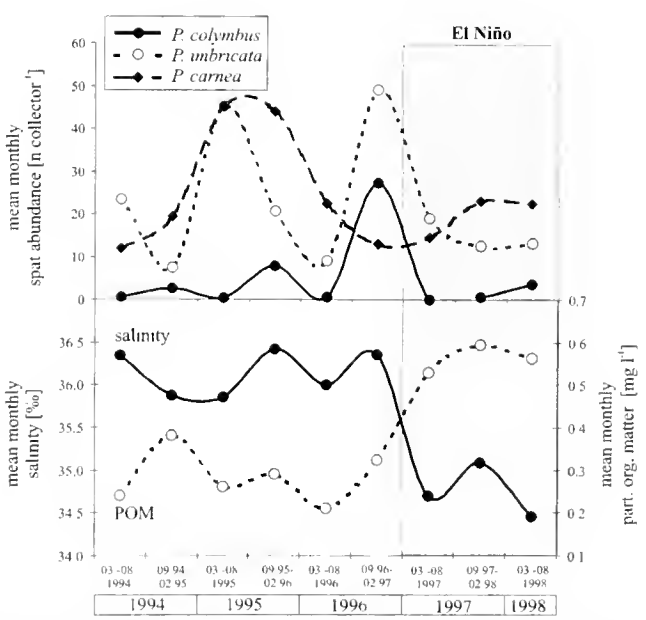

Figure 8. Effects of El Niño on salinity, particulate organic matter and spat abundance of *Pteria colymbus*, *Pinctada imbricata* and *Pinna carnea*. Six months means of original data.

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POPULATION STRUCTURE AND RECRUITMENT OF THE BIVALVE *ARCTICA ISLANDICA* (LINNAEUS, 1767) ON GEORGES BANK FROM 1980–1999

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ABSTRACT *Arctica islandica* is a commercially important bivalve from the North Atlantic Ocean. Due to its slow growth rate and longevity, long-term studies are needed to understand its population dynamics, particularly recruitment. This study describes the spatial distribution and population structure of *A. islandica* on Georges Bank, USA from 1980–1999. Results are based on samples collected with a hydraulic clam dredge during National Marine Fisheries Service ocean shellfish surveys. In all surveys from 1980–1999, individuals were abundant on the south flank of Georges Bank between 60 and 75 m depth. In the 1980s, size distributions in tows were typically unimodal, and the population was dominated by large individuals, 75–90 mm in length. In contrast, bimodal size distributions were observed at many stations in the 1990s, and small individuals (<70 mm in length) often represented 20–50% of the catch (by number). These small individuals were the result of spawning in the 1980s. Ages of clams were estimated from shell banding at one station in 1994. Most individuals had less than 40 bands, and individuals between 21 and 28 were rare. Among young individuals, modes were centered on 7 and 12 bands; assuming band formation is approximately annual, this suggests recruitment by cohorts spawned during 1986 or 1987 and 1981 or 1982. These results imply that annual recruitment of small *A. islandica* on Georges Bank has been highly variable during the last 40 years and suggest an increase in recruitment to the fishery after 1990.

KEY WORDS: *Arctica islandica*, Georges Bank, recruitment, growth, population dynamics

INTRODUCTION

The purpose of this study is to provide a comprehensive analysis of the NMFS research survey data collected on the ocean quahog, *Arctica* (=*Cyprina*) *islandica* (Linnaeus 1767), from Georges Bank, USA between 1980 and 1999. Specific goals were to describe the size and age structure, recruitment, individual growth rate and spatial distribution of *A. islandica*. Previous research on *A. islandica* from Georges Bank includes stock assessments (e.g., NEFSC 1990, 1996, 1998), relationships between age and growth (Ropes & Pyoas 1982), and population genetics (Dahlgren et al. 2000).

Arctica islandica is unusual in having one of the slowest adult growth rates among marine bivalves (Turekian et al. 1975, Thompson et al. 1980a, Thompson et al. 1980b, Murawski et al. 1982, Fritz 1991, Kraus et al. 1992). The species is widely distributed along the coasts of the North Atlantic Ocean (Nicol 1951, Merrill & Ropes 1969, Brey et al. 1990, NEFSC 1998, Witbaard et al. 1999). In the United States, a valuable commercial fishery for this species has existed since the 1970s (Murawski & Serchuk 1989, Serchuk & Murawski 1997, NEFSC 1998). In this fishery, the clams are typically harvested between 80 and 100 mm in shell length (NEFSC 1998). A commercial fishery has also developed recently in Iceland (Thórarinsdóttir 1997). Understanding the population dynamics of slow growing species, particularly recruitment, can provide the information needed to determine rates of sustainable exploitation.

Inherent in the measurement of recruitment is the definition of the term *recruitment*. For purposes of this study, an individual is considered to have recruited when it is large enough to be measured in the National Marine Fisheries Service (NMFS) stock assessment survey (this size is approximately coincident with the age of first reproduction (Thompson et al. 1980b) and the size at which individuals are vulnerable to commercial fishing equipment). This functional (and liberal) definition implies that a recruited individual has survived a host of planktonic, settlement and post-settlement hazards. For comparison, the term *settlement* will be used to describe the arrival of a meroplanktonic larvae onto suitable substrate. In *Arctica islandica* the years between settlement and recruitment involve myriad largely unknown risks and processes. Our interest focuses on developing an understanding of the temporal variation in recruitment to the fishery using the NMFS data.

The National Marine Fisheries Service has carried out standard research surveys of *Arctica islandica* off the US east coast since 1980. With the exception of an area south of Long Island, NY (Murawski et al. 1982), there was little evidence of small (50–70 mm) individuals throughout the 1980s (NEFSC 1998); however, the abundance of clams in this size class has always been uncertain because the clam dredge used to collect the survey samples does not retain all of the individuals below 70 mm in length (NEFSC 1998).

The focal region of this study, Georges Bank, has recently become the focus of a major interdisciplinary research project on interannual variability in biological and physical oceanographic processes (GLOBEC 1992). Georges Bank is unique for *A. islandica* because a commercial ocean quahog fishery has not occurred there. This represents an opportunity to characterize population dynamics in the absence of direct fishing pressure. Fishing pressure on groundfish and sea scallops in this region has been intense (NMFS 1999), however, and is likely to have caused indirect mortality to *A. islandica* and other infaunal species (Witbaard & Klein 1994, Bergman & Van Santbrink 2000).

MATERIALS AND METHODS

Study Organism

Arctica islandica is a filter-feeding bivalve common throughout the cold, coastal waters of the North Atlantic. It is the only extant species in its genus, which dates back to the Cretaceous (Nicol 1951, Abbott 1974). It is found along the North American coast from Cape Hatteras in the south, to Georges Bank, the coast of Maine, and Newfoundland in the north (Merrill and Ropes 1969, NEFSC 1998). No extant populations occur off the coast of Greenland (Jensen 1912, Ockelmann 1958). Along the coast of Europe it is found from the White Sea to the Bay of Cadiz, while

the Arkona Sea is the eastern limit in the Baltic (Nicol 1951, Merrill & Ropes 1969, Brey et al. 1990). The species has also been reported from the coasts of Iceland, the Faroes, the Shetlands, the British Isles and Norway (Nicol 1951, Thórarinsdóttir 1997). Along the U.S. coast, *A. islandica* typically occurs at depths of 30 to 75 meters (NEFSC 1998). It is usually found in sand and sand-shell substrates (Fogarty 1981, Thouzeau et al. 1991a,b, Thórarinsdóttir 1997).

Arctica islandica is one of the longest-lived bivalves on the continental shelf, ages of 50–100 years are common in natural populations, and the maximum reported age is approximately 200 years old (Thompson et al. 1980a,b, Murawski et al. 1982, Fritz 1989, Steingrímsson and Thórarinsdóttir 1995).

Rate of growth is relatively fast until the age of 7 years (Thompson et al. 1980a,b). The growth rate of older individuals is among the slowest known for bivalves, with the exception of certain deep-sea species from soft sediments (Turekian et al. 1975, Thompson et al. 1980a, Murawski et al. 1982, Fritz 1991). Ropes and Pyoas (1982) showed that the growth rate of *A. islandica* is faster on Georges Bank than off the coasts of Long Island, NY, USA and Sable Island, Canada. Field studies have shown that among older individuals, females tend to be larger than males (Ropes et al. 1984, Fritz 1991). In the US Middle Atlantic Bight, age at maturity ranges from 4 to 14 years (Thompson et al. 1980b).

Longevity and growth rate determinations have been based on tagging studies and length frequency modal progression (Murawski et al. 1982), as well as on counts of microscopic growth lines within the shell. Numerous studies indicate that these lines are deposited annually and can be used to obtain a reliable estimate of individual age (Jones 1980, Thompson et al. 1980a, Murawski et al. 1982, Turekian et al. 1982, Ropes et al. 1983, Ropes 1984, Ropes & Jearld 1987, Weidman & Jones 1993, Weidman et al. 1994). The exact determination of age from band counts is not always possible because physical disturbances can cause ocean quahogs to produce additional bands (Turekian et al. 1982, Ropes et al. 1983, Ropes 1984, Kraus et al. 1992) and shell wear may result in loss of bands (Ropes 1984).

Data Collection

A stratified random sampling design was used by the National Marine Fisheries Service in surveys to assess stock biomass and size-structure of *Arctica islandica* on Georges Bank (Fig. 1; NEFSC (1998)). Surveys analyzed in this paper were carried out during the summer months of 1980, 1982, 1983, 1984, 1986, 1989, 1992, 1994, 1997, and 1999. A hydraulic clam dredge, 152 cm wide and weighing 3.2 t, was used to collect all samples. The openings in the body of the dredge are 2.5 × 5.1 cm.

Samples of ocean quahogs were collected during surveys by towing the dredge for five minutes at 2.8 km h^{-1} after it made contact with the bottom. The actual distance towed varied between stations due to differences in station depth and winch speed, which affected time to deploy and retrieve the dredge. Actual distance sampled at each station was determined from doppler readings for surveys conducted from 1980 to 1994, and from bottom contact sensors and inclinometers mounted on the dredge in 1997 and 1999.

For analysis, catch at each station was standardized to a common distance of 278 m (i.e., 0.15 nautical miles), assuming a linear relationship between catch per tow and distance sampled. Given the dredge width, this represents an area sampled of 423 m^2 per

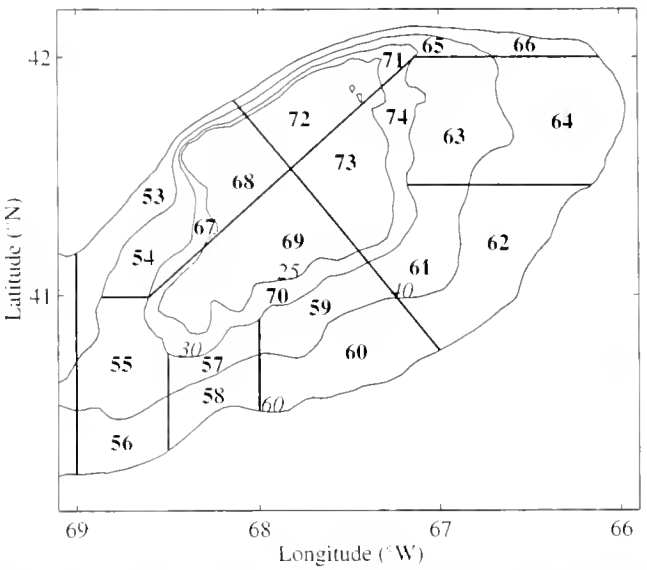

Figure 1. National Marine Fisheries Service ocean shellfish survey strata on Georges Bank. Boundaries of the survey strata are at the 25, 30, 40 and 60 fathom isobaths (1 fathom = 1.83 m). Dotted line indicates the boundary between U.S. and Canadian waters.

standard tow. This is a conservative estimate of the true density, as the dredge is likely to pass over and destroy some clams (Smolowitz & Nulk 1982). In addition, the dredge has only partial retention of ocean quahogs that are <70 mm in shell length (NEFSC 1998, 2000); while specimens much smaller than the mesh size are frequently retained in the sample, their retention is likely to be very unreliable. Additional details about the clam dredge and sensors are given in Smolowitz and Nulk (1982), and NEFSC (1998).

All live ocean quahogs at each station (i.e., tow) were counted and shell lengths, defined as the maximum anterior to posterior distance in mm, were measured at sea to compute average size-frequency distributions per tow for each stratum.

In 1994, a bimodal size structure was noted in several tows on the south flank of Georges Bank (Lewis 1997). The existence of numerous small (<70 mm) clams implied a surge in recruitment to that area. To estimate when these ocean quahogs were spawned, a random subsample of 144 individuals (from a total of 883) was taken from Station 448 in Stratum 61 (41°9′N and 67°1′W) for age estimation based on counts of internal bands. Shell lengths were also measured to estimate growth rate.

The procedure for determining age involved several steps. Each clam was labeled and a valve was selected for sectioning. No preference was given to right or left valves. Shells were sectioned with a glass-cutting saw from the umbo to the most distant portion of the shell. The cut edge was polished on Buehler Polimet Polisher and glued to a microscope slide. A Buehler Isomet 11-1180 Low Speed Saw was used to cut most of the shell away from the slide, leaving a 600–800 μm thin section embedded in epoxy. Sections were ground to 250–300 μm on a Ingram Laboratories Model 305 Thin Section grinder and polished to 150 μm.

To determine clam age, shell bands in prepared sections were counted at least twice per individual using a microscope at ×25 magnification. In the present study, counts of bands were used to indicate age-structure of the population, even though estimated age may not be exact for every individual in the sample. In most cases, an underestimate of age is likely. This is due to the timing and

placement of the year 1 band which is deposited only 3 to 6 months after settlement in a region of the shell that shows heavy abrasion. While not accounted for in our growth calculations, this adds further ambiguity to exact estimates of age.

Age and Length Structure Analysis

A major purpose of the present study was to detect recruitment into the population as indicated by the existence of multiple modes in size- and age-distributions in the NMFS survey data. We used a randomization procedure (Silverman 1981, 1983; Efron & Tibsherani 1993) to statistically test each observed size- and age-distribution for the presence of multiple modes. The test statistic was the minimum value of a smoothing kernel width, h_1 (referred to as "window size"), that would smooth the sampled frequency distribution until there was only one mode or local maximum. A large value of h_1 is required to smooth a distribution with strong and widely separated peaks.

The value of h_1 from the observed data was compared to a probability density function generated by calculating h_1' for each of a large number of bootstrap samples from the original data. The null hypothesis: H_0: number of modes = 1, was rejected if the probability of finding h_1 greater than h_1' was greater than or equal to 0.95.

In cases where the null hypothesis of one mode was rejected, the procedure was repeated, calculating h_2, a window size that generated 2 modes, with a new set of bootstrap samples based on that window size. In general, if the null hypothesis, H_0: number of modes = k, was rejected, then the hypothesis, H_0: number of modes = $k + 1$, was tested.

The exact procedure for the bootstrap involved a number of numerical steps. The minimum value of h such that the function,

$$f(t; h) = \frac{1}{nh} \sum_{i=1}^{n} \phi\left(\frac{t - x_i}{h}\right) \qquad (1)$$

has only one mode or local maximum ($\phi(t)$ is the standard normal density), was determined by successive approximation (values of x_i refer to length, to the nearest mm, or age, in years, of individual clams selected from among the n clams in a given tow). This minimum value was the test statistic, h_1.

Each bootstrap sample contained n individuals drawn at random from the smoothed distribution of the original data following the methods of Efron and Tibshirani (1993). In all tests conducted here, the distribution of h_1' was based upon 200 bootstrap samples. First, n individual values, $y_1' \ldots y_n'$, were sampled, with replacement, from the original data set, $x_1 \ldots x_n$. The values of the n members of the bootstrap sample were calculated from the y' values using the formula:

$$x_i' = \bar{y}' + \sqrt{1 + \frac{h_1^2}{\sigma^2}}\,(y_i' - \bar{y}' + h_1 \epsilon_i); \; i = 1, 2, \ldots, n \qquad (2)$$

where $\bar{y}'$ was the mean of the y' values, σ^2 was the variance of the original sample, x, and the ϵ_i were standard normal random variables. In practice, rather than determine the exact value of h_1 needed to generate a smoothed bootstrap sample with one mode, it was only necessary to know whether h_1' was greater than or less than h_1 for each bootstrap sample. If $P(h_1' > h_1) > 0.05$ (10 out of 200 bootstrap samples), then the null hypothesis was rejected and the alternative hypothesis, that there was more than one mode, was accepted. Otherwise, if $P(h_1' > h_1) \leq 0.05$, the null hypothesis that there was only one mode was accepted.

The statistical test for multiple modes in a frequency distribution was carried out on the observed size-frequency data at each station from Georges Bank. In addition, the test was applied to the observed age-frequency distribution at Station 448 in Stratum 61, defining the x values as the number of shell bands per individual.

RESULTS

Arctica islandica were concentrated around the periphery of Georges Bank, with greatest concentrations on the south flank between 60 and 75 meters depth (Fig. 2 and Fig. 3). Moderate densities of ocean quahogs were found on the steep north flank between 50 and 100 meters depth. Relatively few ocean quahogs were collected from shallower stations located near the center of the Bank (Fig. 3). On the south flank, *A. islandica* were present at almost all stations between 50 and 80 meters depth. The deep water limit for the species was not completely resolved by these surveys, although samples taken at depths greater than 90 meters contained very few individuals (Fig. 2 and Fig. 3).

From the 1980s to the 1990s there were changes in size structure of the population on Georges Bank that were suggestive of an increase in recruitment. In the 1980s, the size structure within most strata was unimodal and dominated by large, 75–90-mm, individuals. In contrast, the size-frequency distributions from 1992 to 1999 contained large individuals as well as numerous (i.e., tens to hundreds) small clams, 40–70 mm in length. The dredge limitations make it difficult to assess the magnitude of the recruitment, but the consistency of the observations over several regions and years provide strong support for the hypothesis that the observations are more than a mere sampling artifact. The data from 1982–1984 and 1994 (Fig. 4) demonstrate typical size frequency distributions from surveys in the 1980s and 1990s, respectively.

The appearance of small individuals in the 1990s was widespread along the south flank of Georges Bank, including Strata 57, 59, 60, and 61 (Fig. 1, Fig. 5, and Fig. 6). This increase in small clams was unlikely to be a sampling artifact because the same dredge, bar spacing, and mesh liner were used throughout the

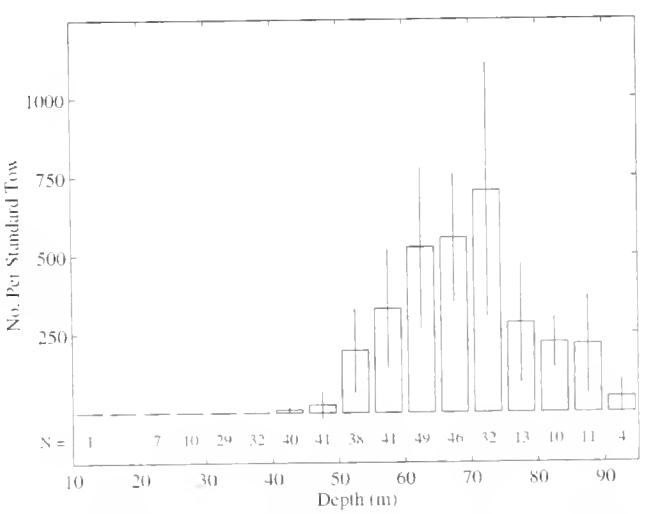

Figure 2. Mean (bar) and 95% confidence interval for number of *Arctica islandica* per standard tow, in five meter depth intervals for all representative tows on Georges Bank from 1980–1999. Number of tows taken within depth intervals is shown at bottom of the bar.

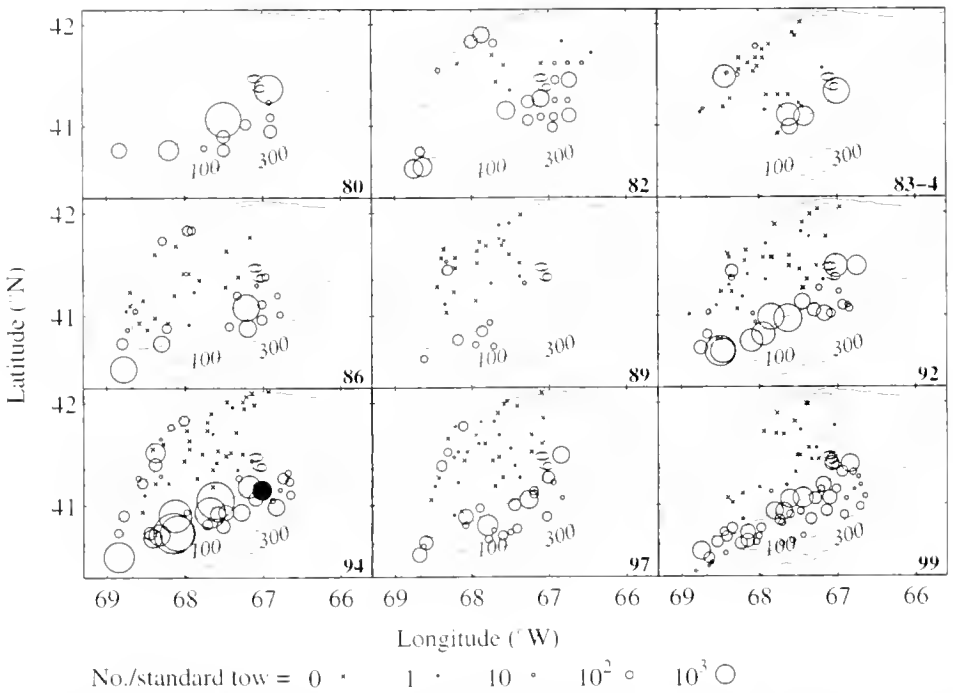

Figure 3. Number of *Arctica islandica* per standard tow from the 1980–1999 survey cruises. Area of circle is proportional to catch, in numbers. The station at which age frequency sampling was performed in 1994 is designated by a filled circle. Isobaths are in meters.

study. In the 1990s, the percentage of small (<70 mm in length) ocean quahogs per sample often ranged from 20–50% (Fig. 6). These percentages are minimum estimates for the actual population structure because the dredge has partial selectivity for ocean quahog shells <70 mm.

The data (Fig. 5) also suggested that level of recruitment varied between strata. For example, a group of small (30–45 mm long) individuals was common in Stratum 59 in 1997, but this group was not as abundant in adjacent Stratum 61.

Statistical tests confirmed that population structure changed over time. Specifically, we tested whether the ocean quahog catch from each tow from 1980 to 1999 satisfied three criteria that suggest recent recruitment. The three criteria were: (1) the statistical test of multimodality had to be significant, (2) at least one of the modes had to be centered below 70 mm in length, and (3) abundance of small (<70 mm) clams had to exceed 20 clams per standard tow. This criterion was for statistical and not ecological significance, making the test more rigorous by eliminating tows that captured a low number of individuals. This represents an abundance far below what would be expected in a stable or expanding population (however, the abundance as measured by the dredge is believed to be less than the actual number in the swept area). Of the 404 tows from all 10 surveys, 71 of the size-frequencies had two or more modes; 59 of those had a lower mode centered below 70 mm, and 34 of those had a sample density of at least 20 small clams per standard tow. Among these 34 tows, 32 (94%) were taken on the south flank between 1992 and 1999 (Fig. 7). Only two of the tows that satisfied all three criteria were collected in the 1980s (Fig. 7). The proportion of tows over the entire bank that satisfied all three criteria went from 0.014 in the 1980s to 0.124 in the 1990s. The proportion of tows that satisfied all three criteria was significantly greater in the 1990s than in the 1980s (χ^2 test: $p < 0.001$). If the statistical test is restricted to data from the south flank (Strata 57–62), where most of the recruitment took place, then the test result is even more significant (Table 2).

The ability to detect recruitment is affected by the intensity of sampling in the 1980s and 1990s. A total of 146 tows from the 1980s were tested for satisfying the three criteria, compared to 258 tows from the 1990s.

The size-based results motivated a study to identify the ages of individuals that recruited to the surveyed population in the 1990s and to infer whether such recruitment was continuous or irregular in time. The sample from Station 448 was composed of two major age groups (Fig. 8), one with individuals younger than 21 years and another with ages of 28 years and greater. Of the 144 clams in the sample, 7 individuals were over 40 years old, ranging in age from 48 to 101 years.

Most of the clams greater than 70 mm in length were at least 11 years old (Fig. 9). This roughly identifies the age at which ocean quahogs from Georges Bank are fully selected (i.e., retained) by the NMFS clam dredge. Extrapolating from the data collected at Station 448, it would appear that most of the clams on Georges Bank that range in length between 70 and 100 mm are 11 to 45 years old. One clam with over 100 bands was found in the sample, but too few clams were sampled in the percent study to determine the maximum age of ocean quahogs on Georges Bank.

To infer which year classes are responsible for the observed recruitment peak in Stratum 61, the test for multiple modes was applied to the age frequency data for ages less than 15 years (Fig. 8). The hypothesis that this group had only one mode was rejected ($p < 0.005$), but the hypothesis that the group had two modes was not rejected ($p < 0.05$). The 2 modes were centered around 7 and 12 bands. Given that the clams were collected in 1994, this would indicate larval settlement by these groups in about 1987 and 1982. Due to the fact that number of bands may not be exactly equal to age (see Methods), we cannot determine from the data whether

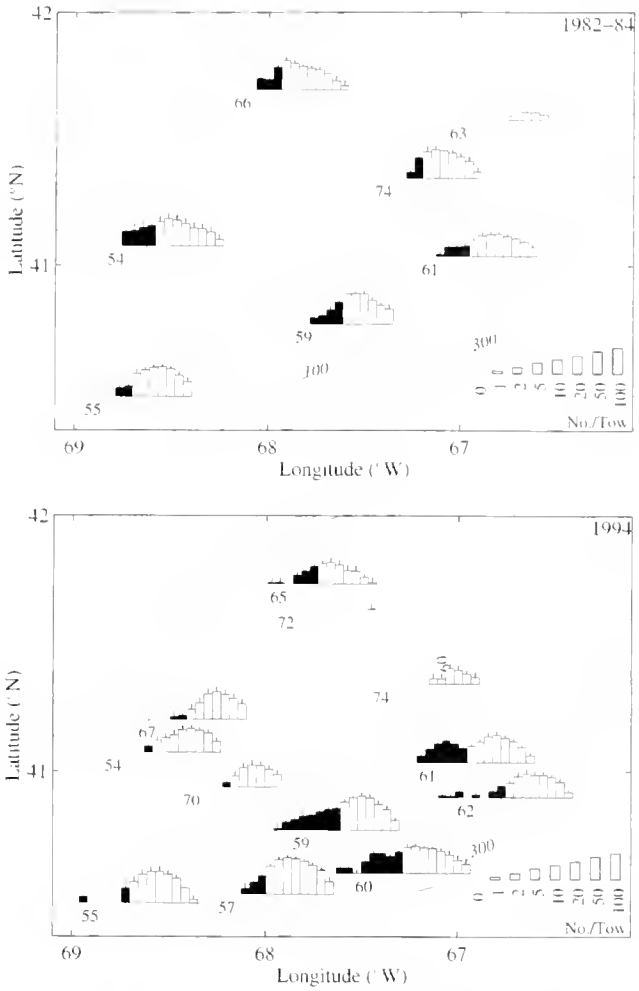

Figure 4. Size structure of *Arctica islandica* by stratum (for strata in which more than one tow was made) over time. Numbers below each histogram denote the stratum number. Individual graphs show the log of the mean (bars) and upper 95% confidence interval (line) for abundance in each size class from 40 to 110 mm, in 5 mm intervals. Size classes smaller than 70 mm are shown in black to highlight the presence of small individuals; dots indicate that no clams in those size classes were recorded.

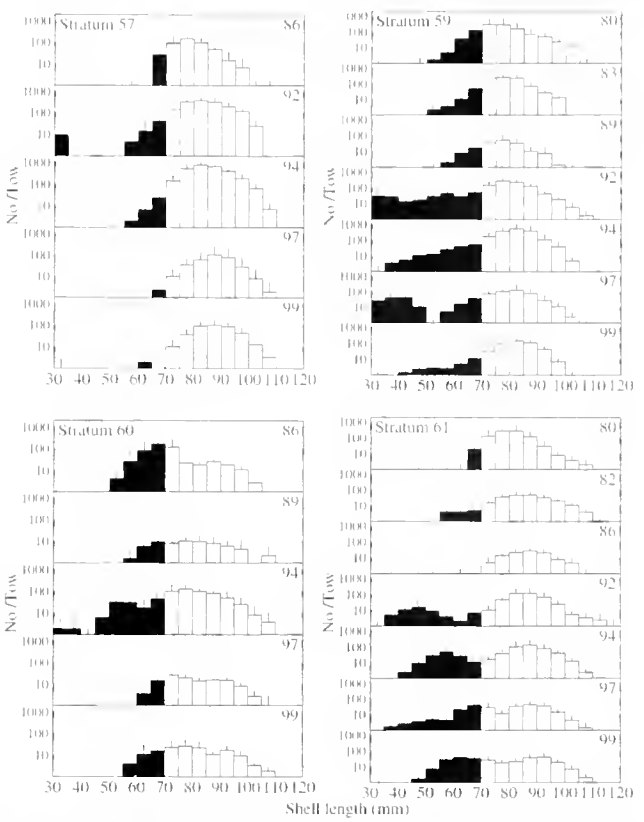

Figure 5. Size structure of *Arctica islandica* in four strata from the south side of Georges Bank, 1980–1999. Graphs show mean (bars) and upper 95% confidence interval (line) for indicated years. Size classes <70 mm are shown in black to highlight the presence of small individuals.

each period of successful settlement lasted for only one year or multiple years.

On Georges Bank, *Arctica islandica* reaches adult size fairly early in its life span (Fig. 10 and Table 1). Individuals grow faster on Georges Bank than in other regions for which growth curves exist. In the 1994 age sample, clams possessing 11–14 bands had an average length of 69.2 mm. The three clams with over 70 bands were smaller than some with a few as 29 bands, implying high variability in growth rate among individuals. An abrupt transition in band structure, from broad (5–10 mm) to narrow (~1 mm) spacing between successive bands, occurred between 7 and 10 bands, implying a shift from relatively rapid juvenile growth to slow adult growth.

DISCUSSION

Several mechanisms may explain the distribution of *Arctica islandica* on Georges Bank between 60 and 90 meters. At these depths, stratification is rarely broken down by tidal mixing. Hence, bottom water temperature is typically cooler and there is lower seasonal variation than in the shallow tidally mixed region within the 60 meter isobath (Flagg 1987). On the south flank, the highest survey catches were recorded near the 60 meter isobath, roughly corresponding to the location of a convergent front that defines the boundaries of the stratified Shelf Water with the tidally mixed Georges Bank Water. This front may serve to accumulate upward swimming larvae at the surface (Mann & Wolf 1983, Franks 1992).

Adult ocean quahogs have low survival at water temperatures greater than 20 C (Turner 1949, Nicol 1951, Landers 1976). Occasional high temperatures in the well-mixed central bank water may be sufficient to exclude *Arctica islandica* from this region. Furthermore, tidal currents over the central bank consistently resuspend sediments which may overwhelm the feeding capability of this filter-feeding bivalve. Finally, competition between *A. islandica* and the Atlantic surfclam, *Spisula solidissima*, may favor the latter in the shallow waters. The distributions of these two species are complementary throughout the Middle Atlantic Bight, with *A. islandica* found in cooler, deeper water (NEFSC 1998). The differing thermal tolerances of the two species are a likely factor in their segregation.

The results suggested that *Arctica islandica* recruit on Georges Bank on an irregular basis. *A. islandica* populations may survive for long periods of time even if recruitment occurs infrequently because of this species' longevity and iteroparous reproductive

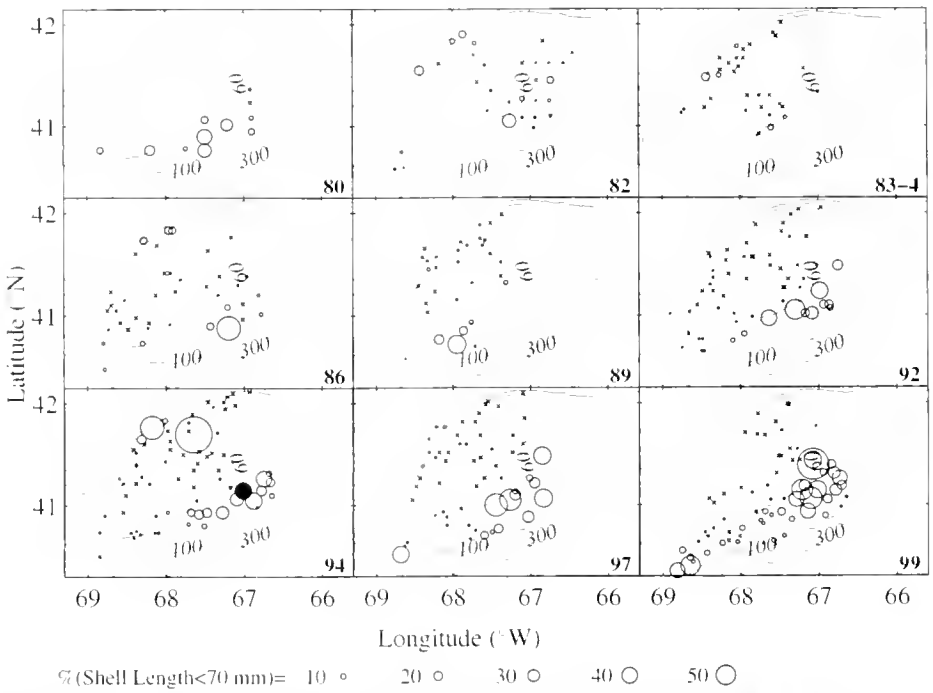

Figure 6. Percent of catch that was smaller than 70 mm in each tow from the 1980–1999 survey cruises. Radius of circle is proportional to percentage (the very large circle in 1994 reflects the presence of a single small clam at the station). Isobaths are in meters.

mode. It is difficult to identify the exact timing and nature of large recruitment events because the ocean quahogs on Georges Bank are not fully retained by the dredge until they are about 70 mm in shell length or about 10 years old. At the station where age-structure was determined, there were apparently 2 major cohorts that settled during the 1980s. In contrast, the absence of individuals between the ages of 21 and 28 in 1994 suggests that little recruitment took place from 1967 to 1974. The high variability in the age structure sample (Fig. 1) also suggests highly intermittent recruitment at this site.

Compared to *Arctica islandica*, shorter-lived and faster-growing bivalves appear to recruit more frequently and with less variation in recruitment strength (Tremblay & Sinclair 1990, Tremblay & Sinclair 1992). Research on two large bivalves from Georges Bank, *Spisula solidissima* and sea scallop (*Placopecten magellanicus*), provides an interesting contrast with *A. islandica*.

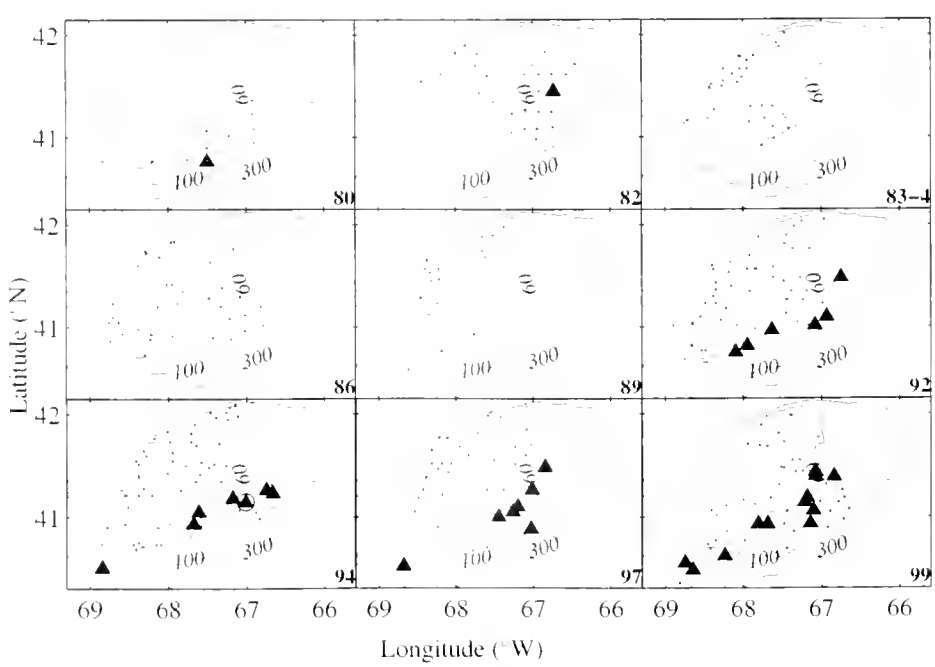

Figure 7. Location of all tows in which two or more modes were detected in the size structure (triangles) for each year, the lowest mode was centered below 70 mm, and density of all individuals less than 70 mm was greater than 20 per standard tow. Isobaths are in meters.

TABLE 1.

Equations for growth curves shown in Figure 10.

Label	Equation	Source
GB94	$L = 97.57(1 - e^{-0.061(a+8.472)})$	Current study, SAS NLIN least squares fit
GB82	$L = 42.4(a)^{0.198}$	Ropes and Pyoas (1982)
MAB	$L = 97.28(1 - e^{-0.031(a+14.967)})$	NEFSC (1990, 1996)
GOM	$L = 59.47(1 - e^{-0.055(a+0.235)})$	Kraus, et al. (1992)

L = length; a = age

S. solidissima has a maximum lifespand of approximately 35 years (Weinberg 1999) and *P. magellanicus* appears to live for 10–15 years (Thouzeau et al. 1991a, Thouzeau et al. 1991b), which makes both species short-lived compared to *A. islandica*. *S. solidissima* recruited successfully along the US Atlantic coast in most years from 1978 to 1997 (Weinberg 1999). Several studies have detected distinct peaks in the size structure of *P. magellanicus* surveyed on Georges Bank, indicative of good annual recruitment (Serchuk et al. 1979, Serchuk and Wigley 1986, Thouzeau et al. 1991a, McGarvey et al. 1993). Given low mortality levels, a long-lived species such as *A. islandica* can maintain viable population levels without recruitment for many years, whereas shorter-lived species require recruitment to prevent extinction.

Many studies report that long-lived species have slow individual growth rates, and exhibit low resilience to and slow recovery from overexploitation (Musick 1999). Although there has been a significant commercial fishery for *Arctica islandica* in the U.S. Middle Atlantic Bight since 1980 (NEFSC 1998), the population on Georges Bank has not been exploited yet commerically. Nevertheless, several major fisheries (e.g., scallops, ground fish) have taken place on Georges Bank throughout the 1980s and 1990s (NMFS 1999). Fish trawls impact the benthic community, causing substantial mortality in bivalves such as *Spisula* sp. and *A. islandica* in the North Sea (Bergman & Van Santbrink 2000). Thus, the changes in population structure of *A. islandica* over time reported in the present study are probably representative of population dynamics under conditions of low to moderate human disturbance.

We have not determined which biological and physical factors control recruitment of *Arctica islandica*. We have examined sea bottom temperature records from Georges Bank (Holzwarth & Mountain 1990), and there is no obvious relationship between observed recruitment and temperature at time of settlement. Other factors which are potentially important include predation, spawning success, availability of larvae, and habitat quality on the sea floor. Potential predators on newly settled clams include various groundfish, such as winter flounder (Steimle et al. 1994), sea stars (Franz et al. 1981), cancer crabs, and the gastropods *Lunatia* and *Busycon*.

Mann (1982, 1985, 1986) provided a complete review of *Arctica islandica*'s larval ecology in southern New England and on Georges Bank. Although the spawning period is prolonged, there may only be narrow windows in time when oceanographic conditions allow for high larval survival and settlement. Mann (1982) hypothesized that phytoplankton becomes available as a major food for *A. islandica* larvae only after wind and storm events mix the stratified water column. It was further suggested that there was

TABLE 2.

χ^2 tests comparing proportion of tows with 2 modes, a lower mode centered below 70 mm and at least 20 clams smaller than 70 mm in the 1980s and 1990s.

Whole Bank			South Flank		
Decade	n_{tows}	$n_{2-modes}$	Decade	$n_{2-modes}$	n_{tows}
1980	146	2	1980	40	2
1990	258	32	1990	88	27
$\chi^2 = 13.49$			$\chi^2 = 67.99$		
$p = 0.00024$			$p < 0.00001$		

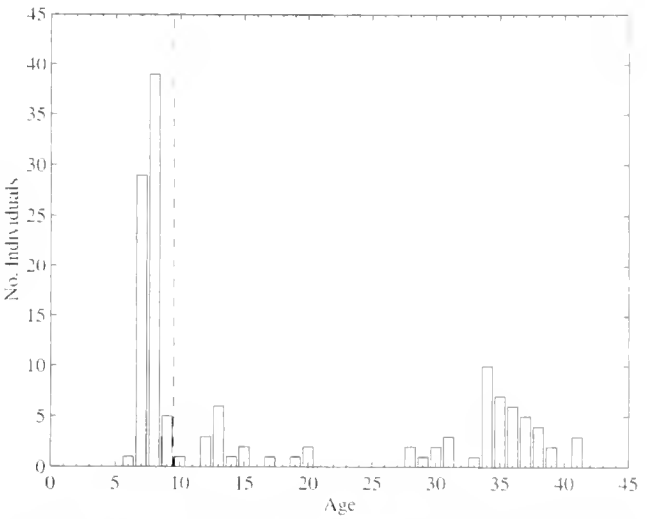

Figure 8. Age structure of ocean quahogs from Station 448 (Stratum 61, 1994 survey), from which a sample of 144 individuals were sectioned for age determination. Dashed line shows approximate minimum age that may to be undersampled by the dredge because of their small size. Seven individuals (not shown) were over 45 years old.

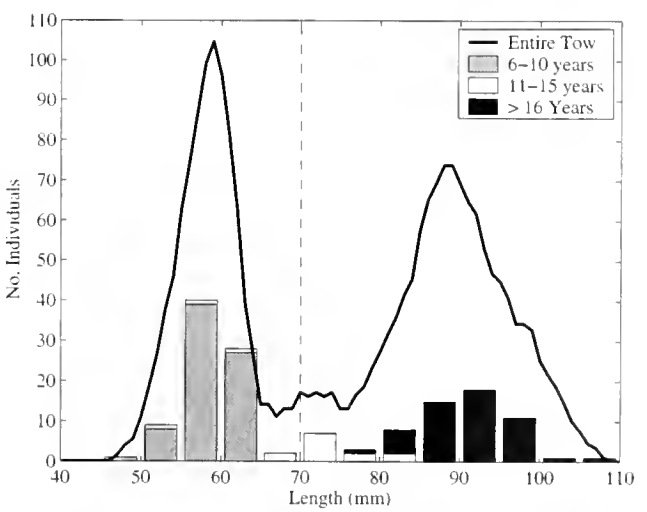

Figure 9. Size structure (for station #448, 1994 clam survey) for all individuals 6–10 (gray), 11–15 (white) and over 16 (black) years old. The shaded bars indicate the size structure of the subsample analyzed for age structure; the dark line shows the measured size distribution of the entire catch (883 individuals) from that tow (smoothed with a 5-mm moving average).

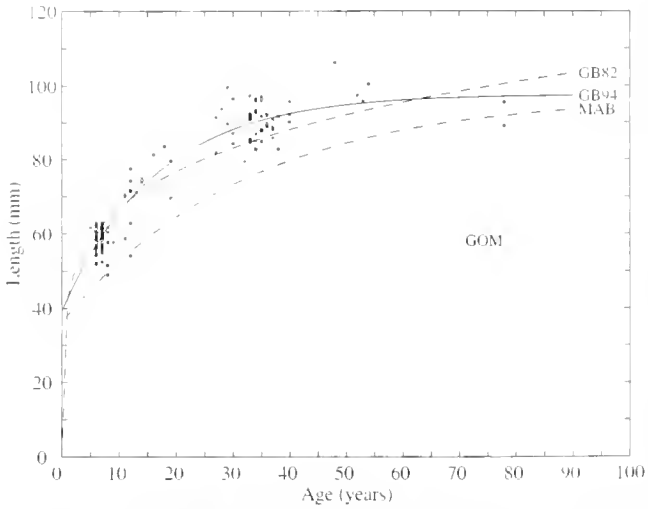

Figure 10. Relationships between age and shell length in *Arctica islandica*. Solid line (GB94): a Von Bertalanffy curve fit to the data ("x") collected in 1994 for the present study of Georges Bank. Dashed line (GB82): a power exponential curve from Ropes and Pyoas (1982) for George Bank. Dash-dot line (MAB) a von Bertalanffy curve from the coast of New York, Middle Atlantic Bight, from NEFSC (1990, 1996). Dotted line (GOM): a curve from a sample in the Gulf of Maine from Kraus et al. (1992). See Table 1 for equations of the growth curves.

no single spawning stimulus; rather temperature, oxygen saturation, pH, and food availability all probably have an effect on spawning time (Mann 1982).

Bottom characteristics vary greatly among different parts of Georges Bank. The south flank, where *Arctica islandica* are plentiful, is composed of poorly sorted gravel, sand and mud. Larger rocks and drifting sand waves, common on the center of the Bank, are relatively rare on the south flank. From a physical standpoint, the south flank is a stable habitat for *A. islandica*.

The population structure of *Arctica islandica* on Georges Bank differs from other regions. For example, compared to populations in the US Middle Atlantic Bight (New Jersey to Virginia), the population on Georges Bank in the 1990s had a higher ratio of small individuals (NEFSC 1998). This is consistent with the results showing that recruitment, as indicated by increased numbers of small individuals in samples, increased on Georges Bank in the 1990s.

Comparisons between growth curves indicate that *Arctica islandica* grows faster on Georges Bank than off the coasts of New York (Murawski et al. 1982), Maine (Kraus et al. 1992), and Iceland (Steingrímsson & Thórarinsdóttir 1995). It is likely that the faster growth of *Arctica islandica* on Georges Bank reflects the region's high productivity (O'Reilly et al. 1987, Backus & Bourne 1987). The experimental work of Kraus et al. (1992) in Maine demonstrated high phenotypic plasticity in the growth rate of this species. In addition, Dahlgren et al. (2000) determined the sequence of the mitochondrial cytochrome b gene of *Arctica islandica* and found little genetic subdivision between Nova Scotia, Canada, and Georges Bank and Virginia, USA. Growth rate in bivalves is affected by many environmental factors, and species may respond differently to these factors due to interspecific differences in physiology and feeding ecology. For example, Weinberg and Helser (1996) compared the growth rate of Atlantic surfclams on Georges Bank with that in other regions along the coast of the U.S. In contrast with *A. islandica, Spisula solidissima* on Georges Bank were smaller at any given age than in warmer regions to the south, closer to the center of *S. solidissima*'s range (Weinberg & Helser 1996).

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SHORT-TERM EFFECTS OF COMMERCIAL CLAM (*MYA ARENARIA* L.) AND WORM (*GLYCERA DIBRANCHIATA* EHLERS) HARVESTING ON SURVIVAL AND GROWTH OF JUVENILES OF THE SOFT-SHELL CLAM

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ABSTRACT In Maine, USA, commercial fisheries for soft-shell clams, *Mya arenaria* L., and blood worms, *Glycera dibranchiata* Ehlers, occur simultaneously on muddy intertidal flats. Local and state clam managers frequently close flats to shellfishing for conservation purposes, but have no jurisdiction over wormers who are legally permitted to harvest *G. dibranchiata* on any intertidal flat. This sometimes causes conflicts, especially when wormers dig in clam conservation areas where clammers have enhanced stocks with wild or cultured "seed" clams (<1 cm shell length, SL). Clammers believe wormers kill or injure small clams directly or indirectly while commercially harvesting *G. dibranchiata*. To help resolve these long-standing conflicts, we worked collaboratively with clammers and wormers and used an experimental approach to test the short-term interactive effects of clam and worm harvesting, harvesting intensity, time of harvest after seeding, and predator exclusion on the fate of small wild and cultured *M. arenaria* at an intertidal mud flat in Brunswick, ME. We added 50 cultured juveniles of *M. arenaria* (SL = 12.5 mm) to 120 1-m^2 plots, 40 of which were undisturbed controls (20 protected with plastic netting—6.4 mm aperture; 20 unprotected) from May to August 1996. The remaining 80 plots were assigned to one of 16 treatments. One half of the plots were protected from predators with the same plastic netting used in the undisturbed control plots. One half of the plots were harvested by a professional wormer or clammer who searched each plot for commercial size blood worms and soft-shell clams, respectively. Plots were harvested either once (after two weeks or four weeks) or twice (two weeks + two weeks, or four weeks + four weeks). Any effect due to clamming or worming on cultured clams or wild individuals of similar size was masked by clam losses exceeding 95% in the unprotected control plots. Intense predation by horseshoe crabs, *Limulus polyphemus* L. and the nemertean worm, *Cerebratulus lacteus* Leidy, are blamed for the high mortalities among clams. Only in protected plots was any effect detected and this depended on clam origin. Compared to the fate of cultured clams in protected controls, worming had no effect, but clamming contributed an additional 15% loss. Both types of commercial harvesting reduced wild clam numbers significantly compared to controls, but effects due to worming were more benign than effects due to clamming probably because wormers excavate less volume of sediments than clammers do as commercial size *G. dibranchiata* are shallow burrowers compared to commercial size *M. arenaria*. Unless clam managers actively take steps to deter predators by using netting or other means, blood wormers should continue to harvest commercially from areas closed to shellfishing without reprisal or fear that they are causing damage to populations of juvenile soft-shell clams.

KEY WORDS: blood worm, commercial harvesting, field experiment, fisheries conflicts, *Glycera dibranchiata*, intertidal, Maine, *Mya arenaria*, soft-shell clam

INTRODUCTION

Soft-bottom, unvegetated intertidal flats throughout the world provide habitat for commercially important infauna such as bivalves and polychaetes (Jackson & James 1979, Emerson et al. 1990, Olive 1992, Brown & Wilson 1997). Harvesting these groups of organisms using hand-held implements such as forks, hoes, rakes, or shovels (Creaser et al. 1983, Wallace 1997) or mechanical devices operated during tidal inundation (van den Heiligenberg 1987, Spencer et al. 1998) erodes or removes sediments to some depth below which the fauna reside. Clam digging, for example, causes sediments to become coarser and to lose significant amounts of organic matter compared with undisturbed intertidal areas (Anderson & Meyer 1986). Effects of turning over sediments on mudflat residents may depend on size and geographic extent of the fishery, sediment grain size, depth to which sediments are excavated, time of year, ability of organisms to evade harvesting or reburrow, or combinations of these factors. When the commercial species are shallow burrowers (e.g., venerid clams such as *Mercenaria mercenaria* [L.] or *Tapes philippinarum* [Adams & Reeve]), local impacts on dynamics of the target fishery such as individual growth and recruitment may not be very long lasting; however, changes in habitat may affect negatively nontargeted infaunal or epifaunal populations (Peterson et al. 1987, Hall & Harding 1997, Spencer et al. 1998). Harvesting deeper burrowing organisms (e.g., polychaetes such as lugworms, *Arenicola marina* [L.] [Cryer et al. 1987, Beukema 1995], and sandworms, *Nereis virens* Sars, [Brown & Wilson 1997], or bivalves such as geoducks, *Panopea abrupta* [Conrad] [Campbell et al. 1998]) can have profound effects not only on the unharvested portion of the population, but also on benthic community structure.

In Maine, two intertidal fisheries occur simultaneously. Soft-shell clams, *Mya arenaria* L., are ubiquitous in soft sediments ranging from gravel and sand to soft mud (B. Beal, pers. obs.). *M. arenaria* is harvested for human consumption and is either processed (shucked) for chowder and fried clam markets or sold live to be eaten after steaming (Ellis & Waterman 1998). In 1999, 1,035 metric tons (t) worth $10.5 million were harvested commercially from Maine flats (NMFS 2001, http://www.st.nmfs.gov/st1/commercial/annual_landings.html). Clams ≥50.8 mm (2 inches) in shell length (SL) are harvested using short-handled hoes with 4 to 6 curved tines that are 30 to 38 cm in length (Robinson & Rowell 1990). The depth that commercial-size animals burrow to is typically ≥15 cm, but this depends on SL (Zwarts & Wanink 1989, Zaklan & Ydenberg 1997) and sediment type (Glude 1954). Blood worms, *Glycera dibranchiata* Ehlers, are typically found in muddy sediments where they are excavated for bait using hoes similar in appearance to those used by clammers, except the tines are shorter, ranging from 20 to 25 cm (Dow & Creaser 1970, Creaser et al. 1983, B. Beal, pers. obs.). In 1999, 233.5 t worth

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$2.9 million were harvested commercially in Maine (NMFS 2001). Although both clammers and wormers ply their trade on muddy flats, digging style differs between the two groups. Clammers usually seek areas of flats where relatively large, oval-shaped holes (that form when animals extend their siphons above the sediment-water interface to feed during tidal inundation) are visible and numerous (B. Beal, pers. obs.). Adults of *M. arenaria* frequently are distributed contagiously (either due to post-settlement processes that tend to move animals toward the upper intertidal [Matthiessen 1963, Emerson 1990] or to harvesting activity that redistributes sub-legal size animals). Clammers, therefore, tend to move from place to place and dig where they notice an abundance of siphon holes. Thus, clam digging is more intense in some portions of the flats than in others. Generally, there is no regular pattern of sediment excavation in the harvested areas (B. Beal, pers. obs.). Conversely, although *G. dibranchiata* can prey on small fauna at or near the sediment surface (Ambrose 1984) and may leave characteristic surface marks or holes (B. Beal, pers. obs.), these are largely ignored by bait worm diggers who collect the worms as they turn over sediments systematically leaving row after row of disturbed mud (Creaser et al. 1983, Brown 1993). Depending on worm density, some flats may be turned over three or more times during the summer months, whereas most clam flats may be turned over twice, but usually only once per year (Brown and Wilson 1997, B. Beal, pers. obs.).

Historically, flats were harvested for worms or clams, not both. Rarely did participants in the two fisheries dig side by side. Flats harvested for worms contained too few commercial size clams or sediments were considered too soft to maneuver within easily for most clammers (D. E. Wallace, pers. comm.). However, as wild clam stocks in Maine fell during the 1980s and price per bushel (approximately 22.7 kg) increased from <$50 to $100 or higher during summer months (Wallace 1997), clammers tended to seek alternative areas that had been long-established harvesting sites for worms. Similarly, the 1980s saw a decline in worm landings in Maine (NMFS 2001) with a concomitant increase in price from $0.04 to $0.06 per worm to nearly $0.10 to $0.15 during summer months (B. Beal, pers. obs.). Wormers, like clammers, began to work outside of their traditionally harvested sites. The result was that both groups began to interact more frequently and, because of large disparities in the management of both species, this caused conflicts between the two groups of fishers.

Clams are comanaged by local stewards elected from each coastal community with a clamming habitat, who work together with officials from Maine's Department of Marine Resources (DMR). Commercial clam harvesting is licensed by both the town and state who together decide annually on the number of licenses to be sold in a particular town (Ellis & Waterman 1998). Today, most communities require clammers to participate in conservation programs prior to obtaining a license. These programs vary from assessing standing stocks or collecting water samples for testing levels of fecal contamination, to transplanting wild "seed" clams from upper to lower intertidal areas or enhancing wild stocks using hatchery-reared soft-shell clam juveniles. Although mandated, conservation programs give clammers a sense of responsibility for, and ownership of, the resource (B. Beal, pers. obs.).

Conversely, worms are not managed by local communities or the state of Maine because their mobility prevents adequate temporal predictions of standing stock (Creaser et al. 1983). Therefore, no management plan exists for blood worms or the other commercial bait worm in Maine, sand worms, except that harvesting must be done using hand-held tools and no Sunday digging is permitted. Wormers may dig on any flat along the coast, and their activities are not restricted by clam management programs. When local clam stewardship committees decide, for example, to institute a conservation closure for rotational purposes or to restock an area with wild or cultured juveniles, there is no legal mechanism to restrict the activities of bait worm diggers in these areas. Conflicts arise because clammers believe bloodworm harvesting kills or injures clams, especially juveniles that occupy the same infaunal depths as the worms. Similar conflicts have occurred from time to time in Maine. In fact, wormer-clammer interactions were discussed and debated by the Maine State Legislature as early as the late 1950s; however, no regulatory action was ever taken as it was decided to allow both groups to settle their own disputes (D. E. Wallace, pers. comm.).

Only one study has examined the effects of worm digging on *M. arenaria* populations in Maine. Ambrose et al. (1998) surveyed number and size of exposed soft-shell clams in freshly dug sediments created by bloodworm harvesters at an intertidal flat in Maine during Fall 1996. They found that approximately 6% of clams were transported to the sediment surface and that of these, 20% had at least one damaged valve. The fate of these clams was not assessed, nor did they examine the fate of animals that may have been excavated and buried within the dug areas. Ambrose et al. (1998) did assess mortality of clams experimentally exposed in several reburial trials and they found that animals placed in recently dug vs. unharvested sediments suffered greater predation risk.

In 1995, the marine resources committee of the southern Maine town of Brunswick enacted a conservation closure (no shellfishing for 1 y) on one of its intertidal flats because hatchery-reared soft-shell clam juveniles had been planted in the spring to enhance wild stocks. During that summer, wormers began commercially harvesting *G. dibranchiata* within the closed area. The local stewardship committee requested, and the commissioner of DMR approved, an emergency closure of the intertidal area to all types of commercial harvesting until such time that a study could be conducted to examine the effects of worming on the fate of small clams. It is rare that adaptive management strategies and experimental approaches are considered by fisheries managers (Botsford et al. 1997, Lenihan & Micheli 2000), but representatives from both fisheries agreed in principle with the approach and requested to be involved with the work.

From May 6 to August 1, 1996, we directly assessed the effects of harvesting commercial size soft-shell clams and bloodworms on the fate of both wild and cultured juveniles of *M. arenaria* at an intertidal flat in Casco Bay (southern Maine) in a small-scale, manipulative field experiment. Specifically, we examined growth and survival of small clams in: (1) unharvested plots either with or without protective, plastic netting; and (2) protected and unprotected plots dug by a professional clammer or wormer at various harvesting intensities. We recommend that bloodwormers not be excluded from areas of the intertidal closed to shellfishing with the objective of enhancing populations of juvenile clams unless a coastal community specifically undertakes direct measures to increase survival rates of the small clams.

MATERIALS AND METHODS

Study Site

The study was conducted from May 6 to August 1, 1996 in Brunswick, Maine near the mid intertidal zone at Maquoit Bay, a shallow (2–3 m at mean high water), semi-enclosed embayment

located in the northeastern corner of Casco Bay (43°51'42"N; 69°59'47"W). Sediments in Maquoit Bay vary in graphic mean size (±1 SE) from the upper (5.29 ± .015 Φ, $n = 2$) to the lower tidal zone (4.95 ± 0.15 Φ, $n = 2$). Salinity and temperature at the site ranged from 30 to 32 psu and 11°C to 19°C, respectively. Approximately 81 hectares of mudflats are exposed twice daily in the bay at mean low water (Heinig & Campbell 1992). An extensive eelgrass meadow (*Zostera marina* L.) existed from immediately below the mid-intertidal to the shallow subtidal and increased in biomass from early May through the summer (B. Beal, pers. obs.). Further features of the hydrography of Maquoit Bay are detailed in Heinig and Campbell (1992). All portions of the intertidal were closed to commercial and recreational shellfish and commercial bloodworm harvesting by the town of Brunswick's marine resources committee and the Maine DMR, respectively, during the study period, and this was effective in keeping both clammers and wormers from harvesting during the experimental period (B. Beal, pers. obs.).

Experimental Design

On May 6, a 1-hectare area near the mid-intertidal zone, approximately 100 m shoreward of the eelgrass meadow, was arbitrarily chosen as an experimental area. This muddy site contained quantities of commercial size *M. arenaria* (≥50.8 mm SL) and *G. dibranchiata* (see Creaser et al. 1983). To estimate initial density and size frequency of soft-shell clams and bloodworms, we took 10 haphazardly placed benthic cores (15 cm deep with an area of 0.0182 m^2) within the hectare and washed each sample through a 3.2-mm sieve. All clams and worms were enumerated and measured (clams – SL; blood worms – width at first setigerous segment; both to the nearest 0.1 mm using vernier calipers).

On the same date, we established 120 1-m^2 plots in a 6 × 20 matrix (2-m spacing between rows and columns) within the experimental area. Fifty hatchery-reared soft-shell clam juveniles (origin: Beals Island Regional Shellfish Hatchery [BIRSH], Beals, $\bar{x}$ SL ± 95% CI = 12.5 ± 0.39 mm, $n = 100$, range = 8.8–20.4 mm) were spread onto the surface of the mudflat in the 1/4-m^2 center of each plot. Small clams typically burrow into muddy sediments within 10 min (Beal 1994). We noted that most *M. arenaria* were not visible after the first 5 min and all had disappeared below the sediment surface after 30 min. Next, 100 of the 120 seeded plots were chosen randomly and covered (protected) with a piece of flexible netting (1.25 × 1.25 m; 6.4-mm aperture; InterNet Corporation, Minneapolis, MN). The remaining (20) seeded plots received no netting. Netting was secured around plots by spreading it over the plot and then forcing the perimeter into the mud to a depth of approximately 15 cm with our feet. A Styrofoam float (10 cm diameter × 4 cm wide) was affixed to the underside center of each piece of netting using a 10-cm-long wooden lath and galvanized nails. Floats enabled each net to rise from its middle above the surface of the flat surface during tidal inundation, permitting clams to filter without disruption (i.e., siphons did not contact the netting). Previous studies (Beal 1994) in muddy sediments indicated that nets without flotation tended to accumulate silt at rates that suffocated, rather than protected, clams beneath. The 20 unprotected plots, together with 20 of the 100 plots that received netting, were used to follow natural mortality and growth of cultured and wild clams in undisturbed sediments through time. These plots served as controls for the larger experiment (see below) that utilized the remaining 80 protected 1-m^2 plots.

On May 20, June 3, July 1, and August 1 (approximately 2, 4, 8, and 12 wk after the experiment was initiated), each of five protected and unprotected (control) plots was sampled ($n = 40$). We used our hands to dig sediments within the entire 1-m^2 to a depth of 15 cm where a hard clay layer existed below which clams on this flat do not burrow (Ambrose et al. 1998, B. Beal, pers. obs.). For the first three sampling dates, each plot was divided into four equal sections to better understand potential clam movement within the 1-m^2 plots. Sediment was sieved through a 3.2-mm mesh, and all cultured and wild clams were enumerated and measured (as above). Hatchery-reared clams are easily distinguished from wild clams due to a unique mark that develops on the shell once individuals are added to sediments (Beal et al. 1999). New shell growth of all living and dead animals (that grew before they died) appears as a white, easily measured band, compared to the darker area near the umbo that forms during crowded hatchery conditions. Other infauna within plots such as milky ribbon worms (*Cerebratulus lacteus* Leidy) and horseshoe crabs (*Limulus polyphemus* L.) were noted and/or counted and, when possible, measured (crabs: greatest carapace width [CW]) to establish size distributions. The five protected and unprotected plots sampled on August 1 served as undisturbed controls for the larger, 2^4 experiment (described below).

The remaining 80 plots protected with netting were assigned randomly to 16 harvesting treatments ($n = 5$) on May 6. A four-factor factorial design was developed after consulting with members of Brunswick's marine resources committee. The experiment was planned to better understand whether differences in survival and growth of wild and cultured juvenile clams could be attributed to the following factors, each with two levels: (1) type of harvesting (bloodworming vs. clamming); (2) rate, or intensity, of digging (plots dug once vs. twice); (3) date of digging (for plots dug once: 2 vs. 4 wk; for plots dug twice: 2 + 2 wk vs. 4 + 4 wk); and (4) protection (plots protected with netting for the duration of the experiment vs. plots with netting removed after final digging; Table 1). A professional clammer using a five-tined clam hoe (as described in Robinson & Rowell 1990) was hired and assigned to harvest (dig) 40 of the 80 plots. The remaining plots were similarly dug by a professional bloodwormer who used a five-tined hoe (as described in Creaser et al. 1983). Neither fisher was chosen by us, but from a pool of local harvesters by their own members.

Twenty of the 40 plots randomly assigned to the clammer were dug once and another 20 plots were dug once by the wormer. Plots were dug either 2 (May 20) or 4 wk (June 3) after the experiment was initiated. The remaining 40 plots (20 clammed and 20 wormed) were dug twice. One group was dug 2 wk after the start of the experiment (May 20) and then again after another 2 wk (June 3). The second group was dug 4 wk after the start of the experiment (June 3) and then again after another 4 wk (July 1).

Netting protected clams in each of the 80 plots until the date when commercial harvesting was scheduled (Table 1). Before digging within any plot, netting was completely removed. After harvesting plots assigned to the "dug once" treatment, one-half were covered again with the same piece of netting, which remained in place until the end of the experiment. The other one-half remained unprotected until the end of the experiment. For plots assigned to the "dug twice" treatment, netting was re-applied to each after the first harvest and it remained in place until the second harvesting. After the second digging, one-half of the plots were covered again with the same piece of netting, while the other one-half were left uncovered (Table 1). (During the study, fishers were given no information about any facet of the experimental design, and neither knew how many, if any, clams had been planted in each 1-m^2 plot.)

TABLE 1.

Experimental treatments initiated on May 6, 1996 and sampled on August 1, 1996 at Maquoit Bay, Brunswick, Maine. $n = 5$.

Treatment	Rate[1]	Date[2]	Protected[3]	Type of Harvesting[4]
1 (Unmeshed control)	CL	C	No	–
2 (Meshed control)	CL	C	Yes	–
3	DO	2	Yes	C
4	DO	2	Yes	W
5	DO	2	No	C
6	DO	2	No	W
7	DO	4	Yes	C
8	DO	4	Yes	W
9	DO	4	No	C
10	DO	4	No	W
11	DT	2 + 2	Yes	C
12	DT	2 + 2	Yes	W
13	DT	2 + 2	No	C
14	DT	2 + 2	No	W
15	DT	4 + 4	Yes	C
16	DT	4 + 4	Yes	W
17	DT	4 + 4	No	C
18	DT	4 + 4	No	W

[1] CL = Control (i.e., not dug); DO = Dug Once; DT = Dug Twice.
[2] 2 = Harvested after 2 wk (May 20); 4 = harvested after 4 wk (June 3); 2 + 2 = harvested after 2 wk and then again 2 wk later (May 20 & June 3); 4 + 4 = harvested after 4 wk and then again 4 wk later (June 3 & July 1).
[3] Plastic mesh netting (6.4-mm aperture).
[4] C = Clammer; W = Wormer.

For plots dug once, fishers were instructed to begin digging 30 cm in from one side of the plot and to dig toward the opposite side (i.e., 70 cm away). This was done to ensure that all excavated sediment would remain within the 1-m^2 plot. Only sediment within the plot was excavated. For plots dug a second time, harvesters were directed to begin digging 30 cm in from the side of the plot where they had last finished digging and to dig toward the opposite side. Again, this harvesting pattern kept all dug sediments within the boundaries of each plot. In addition, each time plots were dug, harvesters were asked to remove all commercial size individuals they noticed of the species each normally would collect. These animals were enumerated and measured (as above). To monitor possible changes in abundance and size of commercial size clams and worms through time, on the last digging date (July 1), each fisher was asked to dig 10 haphazardly chosen 1-m^2 plots outside and adjacent to the 6 × 20 matrix. All commercial worms or clams within these plots were collected, enumerated, and measured (as above).

During the period from July 28 through August 1, each of the 80 harvested plots was sampled as described above, and sediments from each plot were washed through sieves with 3.2-mm apertures. Cultured and wild clams found within each plot were counted and relative growth rate was calculated for each live clam of hatchery origin using the following formula:

$$RG = ([\text{final length} - \text{initial length}]/\text{initial length}) \times 100\%.$$

A random subset of all wild clams from each of the 16 treatments was measured to the nearest 0.1 mm using vernier calipers to estimate final size (SL) frequency.

Statistical Analyses

The design for the 2^4 experiment was a completely randomized one with all four factors fixed (see below). This design yields maximum degrees of freedom to estimate experimental error, and assumes that the area over which the experiment is conducted is homogeneous with respect to biotic and abiotic variables (Steel & Torrie 1980).

Analysis of variance (ANOVA) was used to test all statistical hypotheses. Prior to any analysis, the variance homogeneity and normality assumptions of ANOVA were tested using Cochran's and Shipiro-Wilk's tests, respectively. When significant heteroscedasticity of variances or departure from normality occurred, a transformation was applied to the raw data, and the transformed data were re-tested for variance homogeneity. A source of variation (hypothesis) was considered significant if it was associated with a probability ($P < 0.05$).

A two-way, model I ANOVA was performed on data (arcsine-transformed mean percentage of clam survival, final mean length of hatchery-reared and wild clams, square root-transformed mean number of wild clams) from the control plots where sampling date (a = 4) and netting (b = 2) were the independent variables. A nested, model I ANOVA was performed on similar data from the 2^4 experiment using following linear model:

$$Y_{ijklm} = \mu + A_i + B_j + AB_{ij} + C_k + AC_{ik} + BC_{jk} + ABC_{ijk} + D(C)_{l(k)} + AD(C)_{il(k)} + BD(C)_{jl(k)} + ABD(C)_{ijl(k)} + \epsilon_{m(ijkl)}$$

where: Y_{ijklm} = dependent variable (arcsine-transformed mean percent survival, mean relative growth, square root-transformed mean number of wild clams); μ = overall mean; A_i = type of harvesting (clamming vs. worming); B_j = protection (netting vs. no netting); C_k = rate, or intensity, of harvesting (plots dug once vs. twice); $D(C)_{l(k)}$ = date of harvesting (2 vs. 4 wk; 2 + 2 wk vs. 4 + 4 wk) nested within rate of harvesting; $\epsilon_{m(ijkl)}$ = experimental error—a measure of variation that exists among observations on experimental units treated alike.

Date of harvesting (D_l) is nested within rate of harvesting (C_k) because both rates (dug once vs. dug twice) did not occur on each date when plots were dug (2 vs. 4 wk). That is, plots dug once were only dug after 2 and 4 wk. Plots dug twice were dug on a 2 + 2 wk or 4 + 4 wk basis.

Means, presented graphically or in the text, represent untransformed data ± 95% confidence intervals.

RESULTS

Initial Sampling: May 6, 1996

A mean density of 229 ± 152 individuals/m^2 of *M. arenaria* (SL size range = 5–22 mm) and 0.95 ± 0.55 individuals/m^2 of *G. dibranchiata* (range of greatest width = 3.9–7.9 mm) was found in the 10 benthic cores. The clam, *Macoma balthica* (L.), was the most numerous other large infaunal species within the cores (427.8 ± 395 individuals/m^2) and ranged in size (SL) from 4.2 to 17.4 mm. Although we did find evidence of the nemertean *Cerebratulus lacteus* in most cores, the sieving process damaged the worms too severely and, as a result, we were unable to enumerate or measure individuals.

Control Plots: Hatchery-Reared Clams

Survival of cultured soft-shell clams in both protected and unprotected plots decreased rapidly during the first month of the experiment (Fig. 1). ANOVA indicated that both main factors

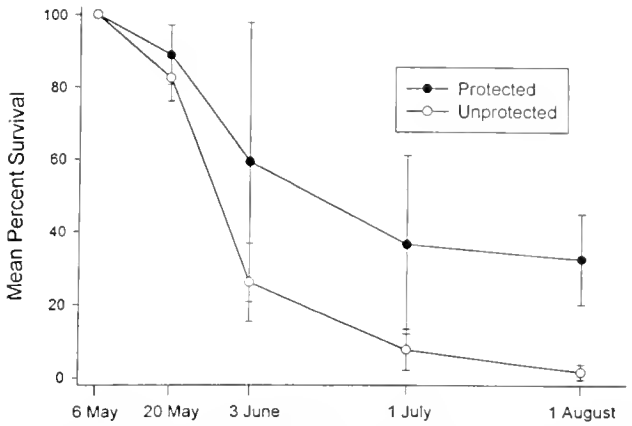

Figure 1. Mean percentage of survival (±95% confidence interval) of hatchery-reared juveniles of *M. arenaria* in 1-m² unharvested (control) plots at Maquoit Bay (Brunswick, ME) from May 6 to August 1, 1996. ANOVA revealed significant clam mortality through time ($P < 0.0001$), and SNK test showed that average survival did not change from July 1 to August 1. Mean survival was consistently ($P < 0.0001$) higher in protected plots. $n = 5$.

(sampling date and level of protection) were highly significant ($P < 0.0001$) and that the differences observed between protected and unprotected plots were consistent through time ($F = 2.14$, df = 3, 32, $P = 0.1140$). An *a posteriori* Student-Newman-Keuls (SNK) test indicated that mean percentage of survival did not decrease significantly after July 1. On August 1, mean clam survival in the plots protected with netting was nearly 20 times greater than survival in plots without protective netting ($\bar{x}_{net} = 32.4 \pm 12.45\%$ vs. $\bar{x}_{no\ net} = 1.6 \pm 2.08\%$; $n = 5$).

The majority of observed mortality (Table 2a) likely was due to processes that kill clams without physically damaging the valves (starvation, disease, and predation by organisms that leave no discernible shell damage) or from predators that crush valves into small (<3.2 mm) fragments. Nearly 90% of clams in the unprotected plots were missing by August 1 compared to 15% in the protected plots (Table 2a). Some missing clams may have been alive, but either had emigrated or been moved from the 1-m² plots by unsuccessful predators. Clam movement, assessed by comparing the number of live animals sampled from the center 1/4-m² areas where they were planted vs. three similar-sized areas within each plot, appeared to increase through time. For example, on the first sampling date (May 20), 98.1% (202/206) and 93.7% (208/222) of live clams were found within the seeded center area of the unprotected and protected plots, respectively. Approximately 8 wk after the experiment was initiated (July 1), 68.4% (13/19) and 75.8% (69/91) of those in unprotected and protected plots, respectively, were found in the area they had been planted.

Only a few clams were found dead with crushed or chipped valves; however, a total of nine *L. polyphemus* was found during the first three sampling dates, and only in plots with protective netting (CW on May 20: 12 and 38.2 mm; June 3: 70.0 and 83.2 mm; July 1: 27.2, 43.8, 46.2, 50.2, and 64.2 mm). The two *L. polyphemus* discovered on June 3 were in a single plot. Only two of 50 hatchery-reared clams were recovered alive from that plot, whereas the other four protected plots sampled on June 3 contained 34, 37, 37, and 38 of 50 clams initially planted. On the final sampling date (August 1), one horseshoe crab was found in one of the five protected plots (23.9 mm) and one occurred in four of five of the unprotected plots (11.1, 32.3, 55.8, and 58.0 mm). A minimum of two *C. lacteus* (approximately 70 mm TL) was found primarily in protected plots on each sampling date. Only one *C. lacteus* (approximately 90 mm TL) was sampled from the unprotected plots (June 3); however, smaller worms (approximately <40 mm) were also present in most of plots on each date. These animals tended to fragment easily during the sieving process, making accurate estimates of their size and density not possible.

Clam growth was unaffected by the presence of netting ($F = 1.83$, df = 1, 28, $P = 0.1867$). Although ANOVA detected a significant increase in mean SL through time ($F = 15.35$, df = 3,

TABLE 2.

a) Mean percentage (±95% confidence interval) of hatchery-reared soft-shell clam juveniles that were alive (A), dead, with undamaged valves (DU), dead, with crushed or chipped valves (DC), or missing (M) from 1-m² plots on four sampling dates. b) Mean number (±95% confidence interval) of wild individuals of the soft-shell clam from the same plots. Fifty hatchery-reared individuals (mean SL = 12.5 mm) were added to each of $n = 5$ protected (plastic mesh netting; 6.4-mm aperture) or unprotected plots on May 6, 1996. $n = 40$.

Treatment	Date	% A	% DU	% DC	% M
a)					
Unprotected	5/20	82.4 (6.4)	3.6 (2.1)	0.0 (0.0)	14.0 (6.3)
Protected	5/20	88.8 (8.2)	4.0 (3.9)	0.0 (0.0)	7.2 (7.9)
Unprotected	6/3	26.0 (10.7)	5.6 (4.1)	0.8 (2.2)	67.6 (9.5)
Protected	6/3	59.2 (38.5)	14.8 (9.7)	0.0 (0.0)	26.0 (47.3)
Unprotected	7/1	7.6 (5.7)	13.2 (10.5)	2.0 (3.5)	77.2 (9.7)
Protected	7/1	36.4 (24.5)	28.8 (20.3)	1.2 (3.3)	33.6 (35.6)
Unprotected	8/1	1.6 (2.2)	11.6 (9.4)	0.0 (0.0)	86.8 (9.2)
Protected	8/1	32.4 (12.5)	52.4 (16.9)	0.0 (0.0)	15.2 (15.3)
b)					
Unprotected	5/20	136.4 (93.6)	1.4 (1.6)	0.0 (0.0)	–
Protected	5/20	223.4 (52.2)	3.8 (2.5)	0.2 (0.6)	–
Unprotected	6/3	33.0 (15.8)	5.0 (3.6)	1.8 (1.9)	–
Protected	6/3	213.4 (133.8)	15.0 (13.6)	12.8 (6.7)	–
Unprotected	7/1	7.4 (4.7)	7.8 (8.1)	5.4 (8.1)	–
Protected	7/1	90.0 (80.2)	14.0 (4.0)	3.8 (3.1)	–
Unprotected	8/1	3.8 (2.5)	15.4 (4.1)	0.0 (0.0)	–
Protected	8/1	162.4 (32.8)	33.6 (19.2)	0.0 (0.0)	–

28, $P < 0.001$), clams grew, on average, only 4.8 ± 1.2 mm (n = 7 plots) over the nearly 3-mo test, attaining a final mean SL on August 1 of 17.3 ± 1.2 mm. The majority of growth occurred during the final month of the experiment (Fig. 2a).

Control Plots: Wild Clams

The mean number of wild clams varied widely through time (Fig. 3). Both main (sampling date and protection) factors and the interaction term were highly significant ($P < 0.001$). Single-factor ANOVAs for each level of protection were performed to facilitate interpretation of clam losses. Clams in both protected and unprotected treatments suffered significant losses through time ($P < 0.01$; Table 2b). SNK tests revealed that in unprotected plots, a significant number of clams disappeared between the first and second sampling date clams, but no significant additional losses occurred after July 1. In plots protected with netting, no significant differences in clam numbers were detected between May 20 and June 3. Significant losses occurred between June 3 and July 1, but

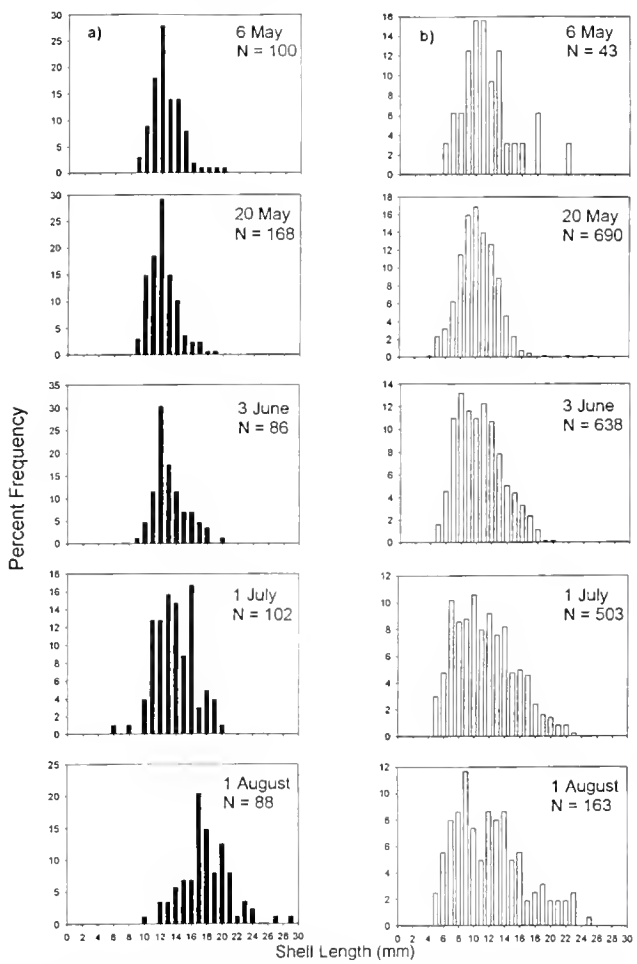

Figure 2. Size frequency distribution of cultured (a) and wild (b) soft-shell clams within 1-m^2 control plots (n = 5) at Maquoit Bay (Brunswick, ME) on each sampling date (except that wild clams on May 6 were sampled from a total of 10 benthic cores [Area = 0.0182 m^2]). Numbers (N) associated with each graph represent total number of clams within plots or cores (except on August 1, where N for the wild clams represents a random sample of taken from 4,386 individuals within 90 plots). ANOVA demonstrated significant increases in mean SL through time for cultured, but not wild, clams.

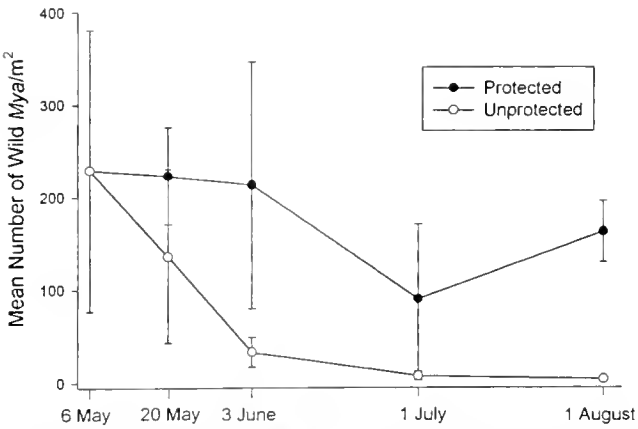

Figure 3. Mean number (±95% confidence interval) of wild individuals of *M. arenaria* within 1-m^2 control plots at Maquoit Bay (Brunswick, ME) on each sampling date (May 20 to August 1, 1996). Initial density was determined from 10 benthic cores taken haphazardly within the experimental area on May 6, 1996. ANOVA performed on data from the 1-m^2 plots indicated both main factors (protection and time) and the interaction of these factors were highly significant ($P < 0.001$). n = 5.

then average numbers appeared to increase to pre-July levels. This variability in mean number may be related to the fact that two horseshoe crabs were found within a single protected plot sampled on June 3 (see above). That replicate contained 104 wild clams, whereas 393, 182, 179, and 209 were sampled from the other four protected plots. In addition, a group of small clams (<5 mm SL) appeared on the final sampling date in the protected plots that may have been too small to be sampled efficiently earlier. These were not 0-y class individuals, as each contained a disturbance ring (winter check) on both valves at 1 to 2 mm SL. By August 1, approximately 40 times the number of wild clams was found in protected plots (162.4 ± 32.85 individuals/m^2) compared with unprotected plots (3.80 ± 2.89 individuals/m^2).

No significant difference in mean SL of wild clams was detected between protected and unprotected plots ($F = 0.67$, df = 1, 32, $P = 0.4191$) nor was there any significant change in mean SL through time ($F = 2.04$, df = 3, 32, $P = 0.1280$; Fig. 2b). The apparent lack of growth also may be related to the appearance of the small clams in samples on August 1.

Movement of wild clams within the 1-m^2 plots was measured indirectly. Since each plot was subdivided into four equal sampling areas for each of the first three sampling dates, we asked whether the distribution of clams observed in the four areas varied through time. We assumed that clam movement occurred if the distribution changed through time. Distribution did not vary significantly in protected ($G = 9.27$, df = 6, $P = 0.1587$) or unprotected ($G = 8.66$, df = 6, $P = 0.1943$) plots.

2^4 Experiment

Hatchery-Reared Clams

Of the 11 sources of variation (hypotheses), the most important (i.e., explaining the largest percent of total variation) associated with percentage of clam survival was that due to protection (meshed vs. unmeshed plots; 51.5%), but this effect depended on the rate plots were dug ($P < 0.0001$; Table 3). Mean survival was higher in protected treatments dug once vs. twice (23.3 ± 3.3% vs.

TABLE 3.

Analysis of variance on the arcsine-transformed percentage survival of hatchery-reared soft-shell clam juveniles within each dug plot after 12 wk (August 1, 1996). $n = 5$.

Source of Variation	df	SS	MS	F	$Pr > F$
Type: Clammed vs. Wormed	1	782.57	782.57	27.71	0.0001
Protect: Mesh vs. Unmesh	1	4,513.95	4,513.95	159.81	0.0001
Type × Protect	1	23.57	23.57	0.83	0.3644
Rate: Dug Once vs. Twice	1	0.38	0.38	0.01	0.9075
Type × Rate	1	0.50	0.50	0.02	0.8945
Protect × Rate	1	700.82	700.82	24.81	0.0001
Type × Protect × Rate	1	39.51	39.51	1.40	0.2413
Date (Rate)	2	47.45	23.72	0.84	0.4364
Type × Date (Rate)	2	31.45	15.73	0.56	0.5758
Protect × Date (Rate)	2	663.18	331.59	11.74	0.0001
Once: (Protect × Date)	1	73.87	73.87	2.62	0.1104
Twice: (Protect × Date)	1	589.31	589.31	20.86	0.0001
Type × Protect × Date (Rate)	2	146.61	73.31	2.60	0.0825
Error	64	1,807.68	28.24		
Total	79	8,757.67			

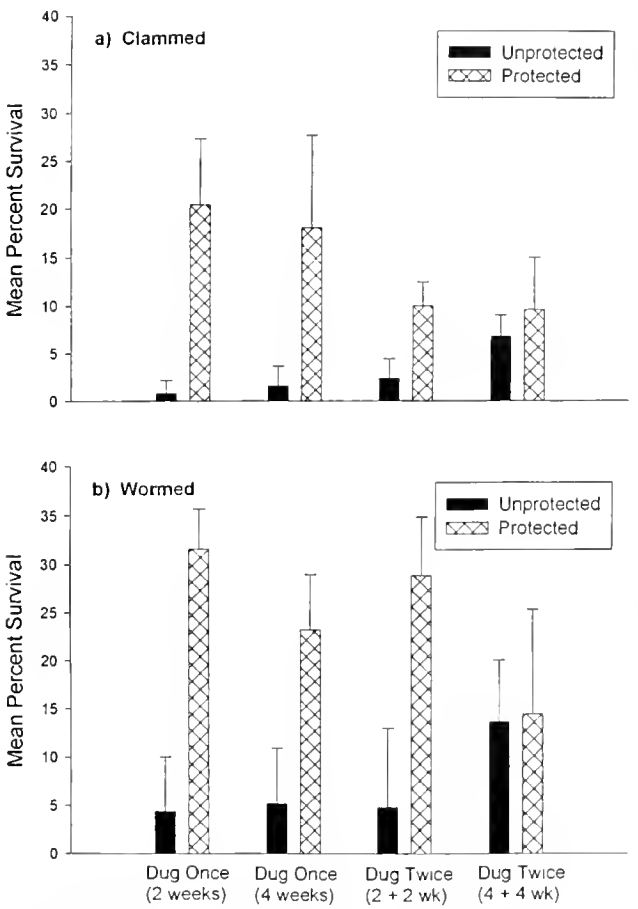

Figure 4. Mean percentage of survival (±95% confidence interval) of cultured soft-shell clams in the eight clammed and eight wormed treatments (Table 1) at Maquoit Bay (Brunswick, ME) on August 1, 1996. Fifty cultured clams (mean SL = 12.5 mm) were placed in the 1/4-m^2 center of $n = 5$ 1-m^2 plots on May 6, 1996. Data represent the percentage of those clams sampled in the entire 1-m^2 plot. ANOVA was used to compare survival between type of fisher, level of protection, intensity of digging, and length of time when digging occurred after the experiment was initiated (see Table 3). $n = 5$.

15.7 ± 4.44%, $n = 20$). This trend was not observed in unprotected treatments where 3.0 ± 1.70% survived in plots dug once vs. 6.9 ± 2.74% in those dug twice. The type of harvesting method, independent of other main or interactive effects, was also highly significant ($P < 0.0001$; Table 3; Fig. 4). Mean survival in clammed treatments was nearly one-half that observed in wormed plots (8.7 ± 2.51% vs.15.75 ± 3.69%, $n = 40$). The only other significant source of variation was that due to Date × Protection (Rate). Decomposing this source of variation into its two orthogonal components revealed that for plots dug twice, the effect of protecting clams with netting, was more pronounced when they were dug on a 2 + 2 wk schedule than when dug on a 4 + 4 wk schedule (Table 3; Fig. 4). This result is not too surprising because unprotected plots dug twice on a 4 + 4 wk basis were actually protected with netting a month longer than animals in the 2 + 2 wk treatments. It also reflects the temporal difference in clam loss observed in the controls between June and July vs. July and August (Table 2a).

TABLE 4.

Analysis of variance on the untransformed relative growth data for living hatchery-reared soft-shell clams within each dug plot after 12 wk (August 1, 1996). Not all plots contained live clams at the end of the experiment, making the data unbalanced. Type III sums of squares were used in all hypothesis tests (Shaw & Mitchell-Olds 1993).

Source of Variation	df	SS	MS	F	$Pr > F$
Type: Clammed vs. Wormed	1	0.0167	0.0167	1.06	0.3086
Protect: Mesh vs. Unmesh	1	0.1597	0.1597	10.14	0.0026
Type × Protect	1	0.0031	0.0031	0.02	0.6587
Rate: Dug Once vs. Twice	1	0.0203	0.0203	1.29	0.2626
Type × Rate	1	0.0348	0.0348	2.21	0.1439
Protect × Rate	1	0.1240	0.1240	7.88	0.0073
Type × Protect × Rate	1	0.0361	0.0361	2.29	0.1370
Date (Rate)	2	0.0376	0.0188	1.19	0.3122
Type × Date (Rate)	2	0.0009	0.0005	0.03	0.9720
Protect × Date (Rate)	2	0.0570	0.0285	1.81	0.1750
Type × Protect × Date (Rate)	2	0.0522	0.0522	1.66	0.2015
Error	46	0.7244	0.0157		
Total	61	1.2668			

To determine whether digging and type of digging generally had an effect on clam survival compared to the undisturbed controls, we conducted two additional, single-factor ANOVAs. The first analysis tested whether mean percentage of survival differed between unprotected controls ($\bar{x}$ = 1.6 ± 2.08%, n = 5) and unprotected, but dug, plots from the larger experiment ($\bar{x}_{clammed}$ = 2.9 ± 1.31%, n = 20; $\bar{x}_{wormed}$ = 7.0 ± 2.92, n = 20). The second analysis tested whether mean percentage of survival differed between protected controls ($\bar{x}$ = 32.4 ± 12.45%, n = 5) and protected, but dug, plots from the larger experiment ($\bar{x}_{clammed}$ ± 3.24%, n = 20; $\bar{x}_{wormed}$ = 24.5 ± 4.00, n = 20). Because these tests were not independent of those conducted previously, we conservatively lowered the type I error rate for each according to Winer et al. (1991). Data in both tests was used twice; therefore, we let n = 2 in the following equation: $\alpha_{adjusted} = 1 - (0.95)^{1/n}$. This yielded an adjusted type I error rate, α = 0.0253. No difference in mean clam survival was detected between the unprotected controls and dug plots (F = 3.64, df = 2, 42, P = 0.0349), but a significant effect due to fisher type was observed among protected treatments (F = 12.21, df = 2, 42, $P < 0.0001$). An *a posteriori* SNK test showed that mean survival in controls and plots dug by the wormer was similar and that both were significantly higher than mean survival of clams in plots dug by the clammer.

Mean relative growth rates were unaffected by type of harvesting, but they did vary interactively with level of protection and harvesting rate (Table 4; Fig. 5). Clams grew approximately 10% slower in unprotected treatments dug once vs. twice (20.97 ± 10.12%, n = 11 vs. 30.26 ± 9.85%, n = 13). Conversely, clams grew nearly 6% faster in protected treatments dug once vs. twice (37.29 ± 3.66%, n = 20 vs. 31.38 ± 6.06%, n = 18). As with the survival analyses, we asked whether growth rates of animals in the controls differed from those in the larger, 2^4 experiment, and we divided this into separate analyses for unprotected and protected treatments. We used the conservative α = 0.0253, as above. Only two of five unprotected control plots contained live clams (RG = 18.43 ± 78.95%) vs. 10 of 20 unprotected clammed (RG = 24.92 ± 14.61%) and 14 of 20 unprotected wormed (RG = 26.77 ± 7.51%) plots. No significant difference in growth rate due to type of fisher was noted (F = 0.24, df = 2, 23, P = 0.7890), but the power of this test was very low (< 0.3). Similarly, for the protected plots, no effect due to type of fisher on relative growth was detected in protected plots ($\bar{x}_{control}$ = 42.38 ± 10.06%, n = 5; $\bar{x}_{clammed}$ = 33.47 ± 5.98%, n = 18; $\bar{x}_{wormed}$ = 35.40 ± 4.18%, n = 20; F = 1.47, df = 2, 40, P = 0.2426), but again, the power to detect differences was low (0.6).

Wild Clams

Wild clams behaved similarly to the hatchery-reared animals (Table 5). Approximately 55% of the total variation in mean number of live individuals was explained by the presence or absence of netting; however, this effect depended on both type (P = 0.0007) and rate of harvesting (P = 0.0001). Protecting clams with netting resulted in more than twice the number of wild clams in plots harvested by the wormer compared with those harvested by the clammer. For example, mean difference in number of wild clams between protected and unprotected treatments was 79.2 ± 21.3 individuals/m^2 in plots harvested by the wormer compared with 35.1 ± 11.69 individuals/m^2 in clammed plots (n = 20; Fig. 6). Application of plastic netting to plots resulted in 2.5 times more individuals from treatments dug once vs. twice. The mean difference in numbers of wild clams between netted and unnetted plots dug once vs. twice was 81.9 ± 22.22 vs. 32.4 ± 13.35 individuals/m^2 (n = 20). In addition, the interaction of type and date of harvesting nested within harvesting rate was significant (P = 0.0389; Table 5). As with hatchery-reared clams, effects due to protecting wild clams with netting were more pronounced (both for clammed and wormed plots – Fig. 6) for the shorter (2 + 2 wk) compared to the longer (4 + 4 wk) harvesting intervals.

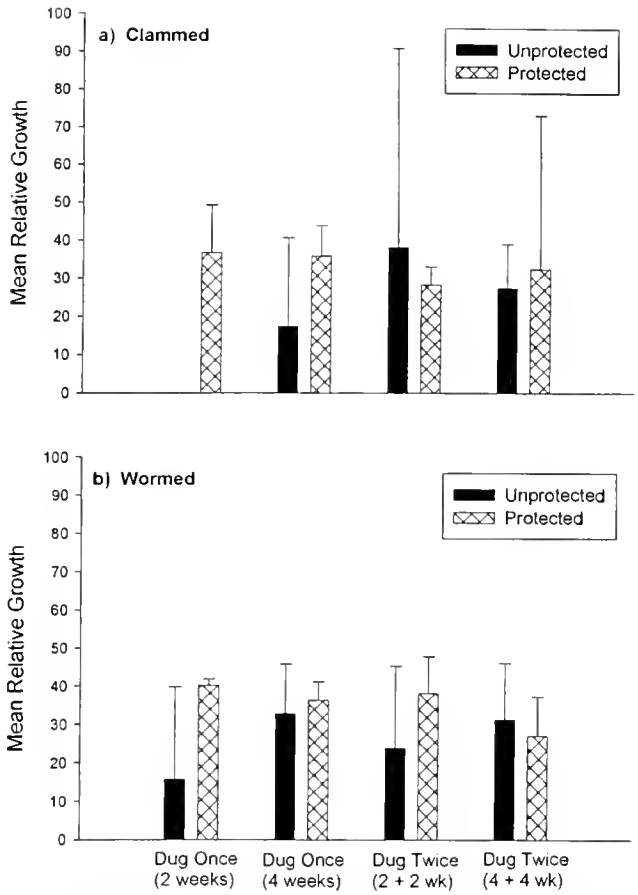

Figure 5. Mean relative growth (±95% confidence interval) of cultured soft-shell clams in the eight clammed and eight wormed treatments (Table 1) at Maquoit Bay (Brunswick, ME) on August 1, 1996. ANOVA was used to compare relative growth between type of fisher, level of protection, intensity of digging, and length of time when digging occurred after the experiment was initiated (see Table 4). n = 5.

We tested whether an overall effect due to digging and digging type existed by comparing, as above, mean number of clams in unprotected and protected controls, separately, to plots treated similarly in the larger, 2^4 experiment. No significant difference ($P > 0.0253$) in mean number was observed in unprotected treatments ($\bar{x}_{control}$ = 3.8 ± 2.39 [n = 5] vs. $\bar{x}_{clammed}$ = 14.1 ± 4.92 [n = 20] vs. $\bar{x}_{wormed}$ = 17.7 ± 7.05 [n = 20]; P = 0.0258). Significantly more wild clams were found in protected controls vs. protected but dug treatments ($\bar{x}_{control}$ = 162.4 ± 32.50 [n = 5] vs. $\bar{x}_{clammed}$ = 49.2 ± 11.74 [n = 20] vs. $\bar{x}_{wormed}$ = 96.9 ± 21.37 [n = 20]; $P < 0.0001$). An SNK test indicated significant differences between all three means.

Commercial Clams and Worms Harvested from 1-m^2 Plots During the Experiment

Experimental plots were dug on three occasions (May 20 = 40; June 3 = 60; July 1 = 20). We asked whether the square root-

TABLE 5.

Analysis of variance on the square root-transformed number of wild soft-shell clams alive within each dug plot after 12 wk (August 1, 1996). $n = 5$.

Source of Variation	df	SS	MS	F	$Pr > F$
Type: Clammed vs. Wormed	1	48.26	48.26	21.58	0.0001
Protect: Mesh vs. Unmesh	1	395.28	395.28	176.77	0.0001
Type × Protect	1	28.67	28.67	12.82	0.0007
Rate: Dug Once vs. Twice	1	6.73	6.73	3.01	0.0876
Type × Rate	1	0.08	0.08	0.04	0.8475
Protect × Rate	1	59.86	59.86	26.77	0.0001
Type × Protect × Rate	1	0.76	0.76	0.34	0.5616
Date (Rate)	2	10.93	5.47	2.44	0.0948
Type × Date (Rate)	2	2.95	1.48	0.66	0.5205
Protect × Date (Rate)	2	15.28	7.64	3.42	0.0389
Once: (Protect × Date)	1	0.86	0.86	0.38	0.5189
Twice: (Protect × Date)	1	14.42	14.42	6.44	0.0136
Type × Protect × Date (Rate)	2	5.57	2.78	1.25	0.2948
Error	64	143.12	2.24		
Total	79	717.49			

transformed mean number and untransformed mean size of animals harvested from each plot varied among sampling dates (Fig. 7). ANOVA detected no significant differences either in mean number ($F = 0.78$, df = 2, 57, $P = 0.4630$) or mean SL ($F = 1.63$, df = 2, 28, $P = 0.2133$) of clams through time. Interestingly, mean number of clams/m^2 adjacent to the experimental site on July 1 (0.9 ± 0.41, $n = 10$) was nearly identical to numbers harvested initially from experimental plots on May 20 (0.95 ± 0.58, $n = 20$). Mean SL of commercial clams within all plots was 64.5 ± 2.16 mm ($n = 39$). No differences were detected in mean number or size of worms per plot from May 20 to July 1 ($F_{number} = 0.88$, df = 2, 57, $P = 0.4205$; $F_{size} = 0.06$, df = 2,34, $P = 0.9385$; Fig. 7). Mean number of bloodworms per 1-m^2 varied little through time. On May 20, 0.95 ± 0.44 individuals ($n = 20$) were harvested from the plots, whereas 1.2 ± 0.74 animals ($n = 10$) were harvested in plots outside the experimental site on July 1. Mean size (greatest diameter) of all worms collected was 5.4 ± 0.32 mm ($n = 37$).

Other Large Infauna Occurring in the Plots at the End of the 2^4 Experiment

The baltic clam, *Macoma balthica*, was the most numerous large infauna within the plots; however, these animals were neither enumerated nor measured. Although horseshoe crabs were found in 50% of the control plots on August 1 (see above), only 15 of the 80 dug plots (18.75%) sampled on that date contained *L. polyphemus*. Horseshoe crabs were found in six of the clammed and nine of the wormed plots. Mean CW of these 24 individuals was 45.5 ± 3.9 mm (range = 10.5–79.8 mm). *C. lacteus* was also found in most protected plots, but again, worms generally were small (approximately < 40 mm TL) and were not enumerated because they tended to fragment easily.

DISCUSSION

This work was intended to investigate the short-term effects of clam and bloodworm harvesting within the soft bottom intertidal on the fate of cultured and wild individuals of the soft-shell clam. Had we not included adequate controls to monitor changes in mean

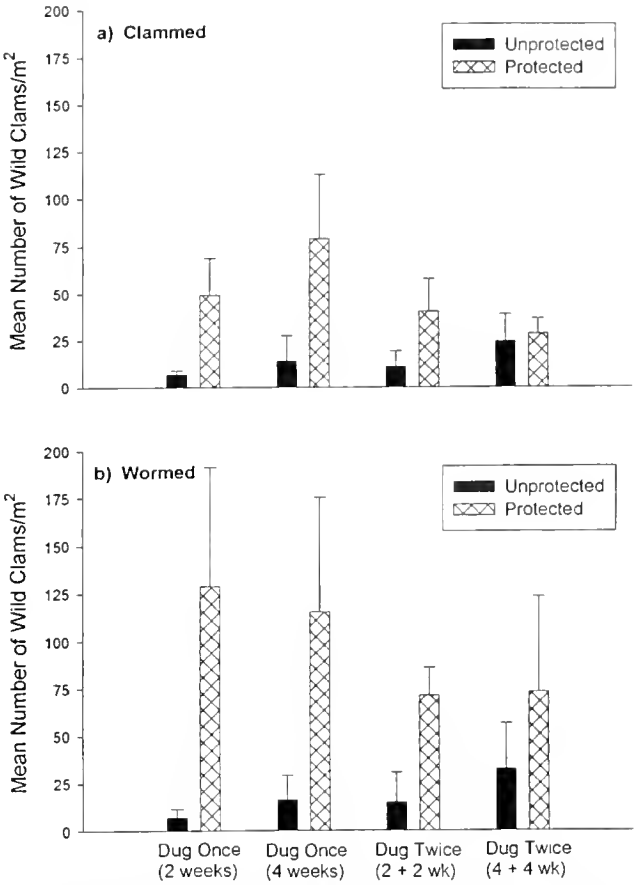

Figure 6. Mean number of wild soft-shell clams (±95% confidence interval) in the eight clammed and eight wormed treatments (Table 1) at Maquoit Bay (Brunswick, ME) on August 1, 1996. Initial density at the beginning of the experiment on May 6, 1996 was 229 ± 152 individuals/m^2. ANOVA was used to compare mean number between type of fisher, level of protection, intensity of digging, and length of time when digging occurred after the experiment was initiated (see Table 5). $n = 5$.

clam survival through time, we likely would have reached a different conclusion about our results. The controls demonstrated clearly that natural losses due to processes that removed clams from the plots (predation and/or emigration) were more important than any effect observed either due to clam or bloodworm harvesting. Each comparison of means between unprotected controls and unprotected, but dug, treatments in the 2^4 experiment was not significant. Had our ability to detect differences between means not been reduced by the necessity to adjust the type I error rate of these tests, we would have concluded that survival of cultured clams and final mean number of wild clams was actually higher in the dug vs. control plots. That is, there might be some benefit to turning over the sediments. Only when we examined the fate of clams protected by netting could we discern any effects due to type of harvesting. The ANOVAs demonstrated that at least for hatchery-reared clams, effects due to worming on mean growth and survival were negligible compared to controls. Clamming, on the other hand, contributed an additional 15% loss of juvenile clams compared to controls. We did detect a negative effect due to bloodworm harvesting on numbers of wild clams in protected plots, but the effect was not as severe as that from clamming. Approximately 40% and 70% fewer wild clams were found in plots harvested by

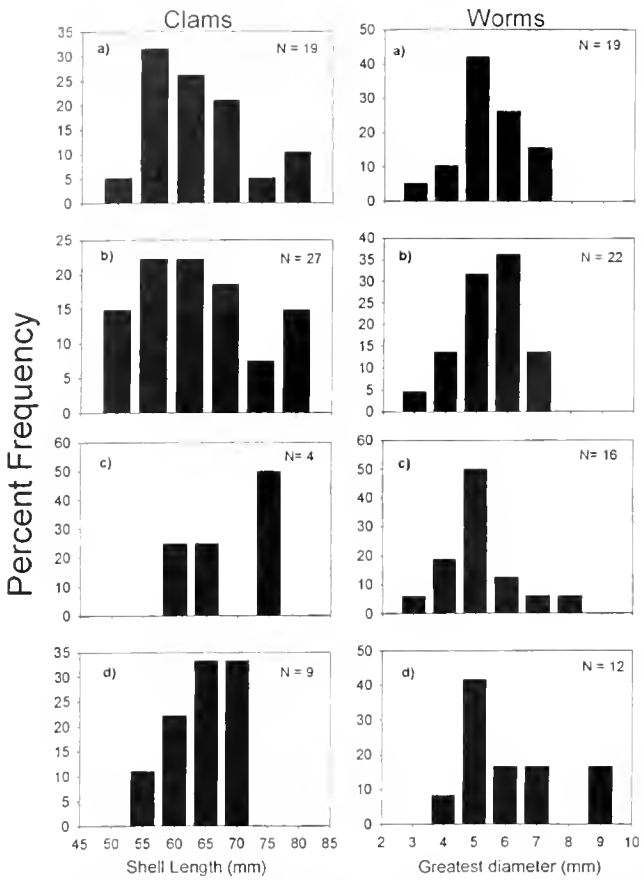

Figure 7. Size frequency distribution of commercial size soft-shell clams and blood worms collected by the clammer and wormer, respectively, from 1-m² plots from the 2^4 experiment at Maquoit Bay (Brunswick, ME) in 1996. a) May 20 (n = 20); b) June 3 (n = 30); c) July 1 (n = 10). Distributions represented in d were taken on July 1 (n = 10) outside and adjacent to the 6 × 20 experimental matrix. Mean SL of *Mya* (64.5 ± 2.16 mm; n = 39) and mean width of *Glycera* (5.4 ± 0.32 mm; n = 37) did not change through time nor did density ($P > 0.05$).

the bloodwormer and clammer, respectively, compared to protected controls.

Results from the 2^4 experiment alone suggest that clamming had the greater negative impact on clam survival than bloodworming (Tables 3 and 5; Figs. 4 and 6); however, we did not examine the mechanism. Observed differences in survival of cultured clams and numbers of wild clams between clammed and wormed treatments may be related to the depth that harvesters must dig to find commercial size animals. For example, commercial size *M. arenaria* burrow ≥15 cm (Zwarts & Wanink 1989) compared to 5 to 10 cm for *Glycera* (Creaser et al. 1983). The tools each type of fisher uses also reflects these life history traits. That is, the tines of clam hoes are significantly longer than tines on hoes used by wormers; therefore, the volume of sediment excavated when searching for clams vs. worms is greater (B. Beal, pers. obs.). Since clam survival is inversely proportional to burial depth (Glude 1954), we infer that clam harvesting adds synergistically to the already large losses of small clams due to natural causes.

Hatchery-reared clams used in this study were in the range of sizes of relatively mobile individuals (Baptist 1955, Beal, 1994). One question might be that since no barriers or enclosure walls (*sensu* Peterson & Beal 1989) were used to restrict clam movements, how is it possible to distinguish the difference between emigration from unprotected plots and clam mortality? We seeded small, cultured clams into the middle 1/4-m² of each 1-m² plot, while the remaining area around it was used as a buffer zone for emigrants. We assume that were emigration important in explaining the relatively high percentage of clams missing from the unprotected controls (Table 2a), most movement from the plots would have occurred within the first 2 wk (May 6 to 20) after plots were seeded. On the first sampling date (May 20), 14% of cultured clams were missing within the five unprotected control plots. Of the remaining 83% of living hatchery-reared clams, 98% were sampled from the middle 1/4-m² of each plot. In addition, although clam movement in protected controls was restricted to the 1-m² area, we found 94% of live animals to be within the seeded middle area on the first sampling date. These data suggest that if clams did emigrate from the 1-m² plots, the rate was very low. In addition, comparable data showed that wild clams had moved very little.

The severe losses of hatchery-reared clams in the controls (protected = 68%; unprotected = 98%) mirrored large losses of small wild clams in the same plots (protected = 27%; unprotected = 97%). Numbers of wild individuals in protected controls appeared to increase from July 1 to August 1, but this was likely due to a combination of two events. The first was a group of small clams (<5 mm) that apparently had grown into the size range sampled more efficiently by the 3.2-mm mesh. The second was predation. It is reasonable to conclude that the majority of clams lost from control plots was due to intense predation. Large predators such as horseshoe crabs were noted on each sampling date. The best evidence that predation occurred and was an important factor in explaining the high percentage of clam losses came from a single protected plot on June 3. In four of five protected plots, the mean number of live hatchery-reared clams recovered out of 50 planted was 36.5, or 73%, and the mean number of wild clams was 241. Two juveniles of *L. polyphemus* were found in the fifth plot that contained only 2 of 50 (4%) living, cultured clams (the rest were missing) and 104 (or 57% fewer) wild clams.

Another line of evidence that predation was important was the number of nemertean worms observed in the control samples, as well as from the larger experiment. Rowell and Woo (1990) provided the first experimental evidence from both field and laboratory trials that *C. lacteus* consumes individuals of *M. arenaria*. These worms leave no apparent damage to the valves such as crushing, chipping, or drilled holes commonly associated with crustaceans and naticid gastropods, respectively. *C. lacteus* gains entry to prey such as *M. arenaria* by wriggling its whole body or proboscis through either the siphons or the anterior pedal hole in the mantle (Kalin 1984). Since cytolytic and neurotoxic proteins are localized in the integumentary tissues of the body wall and proboscis of this predator (Kem 1985, Barnham et al. 1997), it is able to kill its prey without damage to the valves. In this study, numbers of dead, undamaged valves of both hatchery-reared and wild clams in control plots were high (Table 2a and b) and may have been related to nemertean worm predation.

Stress due to mishandling cultured clams and/or disease of wild or cultured individuals are alternative hypotheses for explaining the high proportion of dead, undamaged valves; however, these seem unlikely. Clams (8–20 mm SL) obtained from BIRSH were produced in 1995 and held over the winter (see B. Beal et al. 1995). Survival varied between 95% and 98% (B. Beal, pers. obs.). Hatchery-reared individuals within this range of sizes also were used in a manipulative field experiment initiated in April 1996 on

an intertidal mud flat in eastern Maine (Jonesport; latitude 44°37'N, 67°34'W). In that study clams were planted at densities of 666, 1,332, and 2,664/m^2 in protected and unprotected experimental units at each of three distinct tidal heights. Units (n = 180) sampled on June 5 and August 3, 1996 contained 94.2% and 86.4% live clams, respectively (Beal et al. 2001). No *C. lacteus* or *L. polyphemus* occured on this flat (B. Beal, pers. obs.). Since clams used in both studies were of the same origin and were handled similarly prior to experimental manipulation, it is unlikely that stress due to handling was a significant factor in soft-shell clam mortality in the present study. In addition, although not specifically tested here, since 1992, cultured soft-shell clam juveniles from BIRSH have been routinely (annually) examined histologically for gonadal (B. Barber, University of Maine, Orono, pers. comm.) and hemocytic (S. McLaughlin, NOAA, Cooperative Oxford Laboratory, Oxford, MD, pers. comm.) neoplasia prior to shipping seed out of state. No prevalence of either type of neoplastic cell has been reported.

The level of protection was the most important source of variation in each of the three ANOVAs associated with the 2^4 experiment (Tables 3–5), but in each case, the Protect × Rate interaction term was significant. Wild and cultured clams were found in greater abundance within protected vs. unprotected plots regardless of whether plots were dug once or twice; however, the effect due to netting was greater when plots were dug once vs. twice. This fact, and the failure of ANOVA to detect an effect of rate of digging alone, was partially a result of the experimental design, as unprotected plots dug twice were covered with netting during the time between harvests. In the case of treatments 15 through 18 (4 + 4 wk; Table 1), there is little difference in means between protected and unprotected treatments (Figs. 4 and 6). In protected plots, cultured clam survival was 8% higher, and 43% more wild clams were found in plots dug once vs. twice. When we reanalyzed data from Tables 3 and 5 by ignoring all information from unprotected plots, these percentages were highly significant ($F_{survival}$ = 19.44, df = 1, 32, $P < 0.0001$; F_{number} = 17.50, df = 1, 32, $P < 0.0001$) and suggested that repeated digging of flats results in significant additional clam losses. The presence of netting enhanced growth of cultured clams by nearly 17% in treatments dug once, but this same effect was not observed in treatments dug twice. Again, this may be related to the extra time that unprotected plots were covered with netting. Glock and Chew (1979) found similar growth enhancement in Japanese littlenecks, *Venerupis japonica* (DeShayes), at an intertidal flat in Washington state; however, previous field trials in eastern Maine using cultured seed of *M. arenaria* (Beal 1993) yielded no differences in clam growth between netted and unnetted experimental units.

Except for Ambrose et al. (1998), previous studies on impacts of digging soft-bottom sediments on the fate of soft-shell clams have focused directly or indirectly on effects caused by clammers (Glude 1954, Medcof & MacPhail 1964, Emerson et al. 1990, Robinson & Rowell 1990). Depending on sediment type, harvesting can be 60% to 85% efficient and can also damage or kill animals missed or are left behind intentionally. Unharvested clams may be exposed on the sediment, increasing their risk to predation by birds, fish, and crustaceans, or may be buried at depths that cause them to suffocate. Glude (1954) conducted field experiments in Maine to assess the effects of burial on soft-shell clams (9.5–50.8 mm SL) in muddy sediments. With siphons oriented in three positions (upright, horizontal, and upside down), clams were buried at depths from 2.5 to 23 cm. Survival was related directly to clam size, burial position, and depth. The range of estimates of direct mortality of clams from hoes or forks varies between 2% and 50% (Dow et al. 1954, Medcof & MacPhail 1964, Hruby 1982, Robinson & Rowell 1990). Both Dow et al. (1954) and Robinson and Rowell (1990) determined that breakage (chipped or cracked valves) of clams (13–57 mm) by hoes in commercially productive areas averaged between 17.6% and 19.6%. These means increased with increasing clam density and sediment compaction. Dow et al. (1954) showed that breakage increases with increasing clam size, but Robinson and Rowell (1990) found no clear trend between mortality rate and clam size. Robinson and Rowell (1990) concluded that incidental mortality of clams due to harvesting could significantly affect clam stocks by decreasing yields.

The work by Ambrose et al. (1998) represents the first examination of bait worm digging on soft-shell clam populations. They concluded that commercial harvesting of *G. dibranchiata* has negative effects on clam abundance and that these effects should be considered in state and local clam management programs. Experimental evidence presented here supports their recommendation. We conclude that clamming and worming both contribute negatively to the loss of small soft-shell clams. However, these losses are considered unimportant during the summer months when natural mortality is high due to predation and other sources, especially in areas where netting is not applied to intertidal flats to protect clams from predators or to encourage recruitment of wild seed clams (Beal 1993). Our study and that of Ambrose et al. (1998) was a short-term trial conducted at a single-study site. Although it is dangerous to extrapolate results without spatial or temporal replication, our collective experience since the mid-1980s working with coastal communities in Maine to enhance flats with hatchery-reared juveniles of *M. arenaria* is that the rate of natural predation observed in this study is not unusual for intertidal areas along the southern Maine coast. In the absence of direct measures to enhance juvenile clam survivorship during summer months when natural predation rates are high (i.e., applying protective netting to portions of the intertidal or modifying sediments using gravel or shell [e.g., Toba et al., 1992]), we recommend that local and state managers permit bloodwormers to harvest *G. dibranchiata* from flats closed for shellfish conservation purposes. If the objective of the closure is to protect large individuals (>30 mm SL), we recommend that managers review the work of Glude (1954) and Ambrose et al. (1998). Furthermore, limiting clamming in shellfish conservation areas where natural predation rates are high will likely not result in enhanced densities of small clams. Conservation closures for shellfish are more likely to benefit clams larger than 30 to 35 mm SL; that is, those individuals that have escaped the majority of threats by predators due to their relatively large size that enables them to burrow deeper in the sediments (Commito 1982).

Conflicts between various fisher groups who harvest intertidal organisms in Maine and elsewhere are likely to increase in future as prices for dwindling supplies of natural stocks of commercial marine species continue to increase against a backdrop of conditions that restrict or limit access to fisheries (e.g., management and/or rule-making decisions, local, state, and federal zoning laws, property rights issues, and aquaculture). Recently, it has become popular for fisheries managers to move toward decentralizing decision making and to spread out this process among communities, fishermen, and marine scientists (Wilson et al. 1994). Experimental approaches can be useful tools in helping to resolve ambiguities

and innuendos surrounding gear and other fisheries conflicts (Peterson et al. 1987, McAllister & Peterman 1992, Lenihan & Micheli 2000). This manipulative experiment demonstrated that local clam stewardship committees have a tool available to them to enhance survivorship of juvenile soft-shell clams. If adopted, this approach would necessitate short-term restriction of all types of digging of mud flats in fenced or netted areas during that time of year when clam predation and other sources of mortality are greatest. Conversely, if no attempts are made to deter predation on small clams, no changes in current rules and regulations are warranted.

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GENETIC VARIATION IN POPULATIONS OF THE COMMON CHINESE CUTTLEFISH *SEPIELLA MAINDRONI* (MOLLUSCA: CEPHALOPODA) USING ALLOZYMES AND MITOCHONDRIAL DNA SEQUENCE ANALYSIS

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ABSTRACT Allozymes and sequence of cytochrome oxidase subunit I (COI) gene were used to investigate the genetic variation in five populations of the common Chinese cuttlefish *Sepiella maindroni* in waters of China and Japan. Samples were examined at 14 enzymes comprising 23 putative allozyme loci. The results revealed moderate levels of genetic variability: the proportion of polymorphic loci = $0.226_{(0.99)}$, mean observed heterozygosities per locus = 0.038, mean expected heterozygosity per locus = 0.039, and the average effective number of alleles = 1.26. Nei's mean genetic distance ranged from 0.0001 to 0.0018.

Part of the cytochrome oxidase subunit I gene was amplified with the polymerase chain reaction (PCR) and sequenced for 27 individuals: five to six from each of the five populations. Sequence data showed that there were 11 variable nucleotide positions in the 661 base-pair segments of the gene, and the 27 sequences could be grouped into nine haplotypes (A–I). No remarkable genetic difference was observed among those populations.

KEY WORDS: genetic variation, *Sepiella maindroni*, allozyme, cytochrome oxidase I gene, DNA sequencing

INTRODUCTION

The cuttlefish *Sepiella maindroni* de Rochebrune, 1884, is widely distributed in Asia. It is an important and valuable fishery resource in China, Japan and South Korea (Dong 1991, Nesis 1987, Okutani 1995). Its output accounted up to about 60% of the total cephalopod production in 1980s in China, and occupies an important position in Chinese marine products (Qi 1998). But its yield has declined severely since then, due to over-fishing. One of the main reasons for the decline of population size is that aspects of its population biology are poorly documented, especially the population structure and genetic diversity.

Allozyme electrophoresis can provide direct information on the distribution of genotypes in accordance with the Hardy-Weinberg paradigm. It remains a valuable approach to describe breeding structure of a species (Carvalho & Nigmatullin 1998). Few genetic studies have been carried out on cuttlefishes, and all showed low levels of polymorphism (Pérez-Losada et al. 1996, Pérez-Losada et al. 1999). For providing useful information for resource management and protection, we examined four populations in the coastal waters of China, and one sample from Nagasaki port in Japan using allozyme electrophoresis. Intraspecific sequence variation within mtDNA has also proven a powerful tool for examining population structure in marine organisms (Carvalho & Pitcher 1994). The combination of mitochondrial DNA and allozyme analysis may be helpful in understanding the evolutionary dynamics of these interacting populations of marine species (Hilbish 1996). We further investigated genetic constitution of those populations through examining nucleotide sequence of part of the cytochrome oxidase subunit I (COI).

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MATERIALS AND METHODS

Samples

Fresh samples were randomly collected from five locations in the waters around China and Japan (Fig. 1, Table1), and kept frozen at −20°C until dissection. Mantle length of all the specimens was measured and sex were recorded (Table 2). Mantle muscle tissue, liver, eyes and buccal muscle were taken from each individual in ice as quickly as possible, then transferred for storage at −80 C allozymic analyses. Muscle samples were removed and kept in liquid nitrogen for DNA analysis.

Allozymic Analysis

Electrophoresis was carried out using standard horizontal starch gel techniques (Harris & Hopkinson 1976), essentially as described by Zheng et al. (2001b). Forty-six putative enzyme-

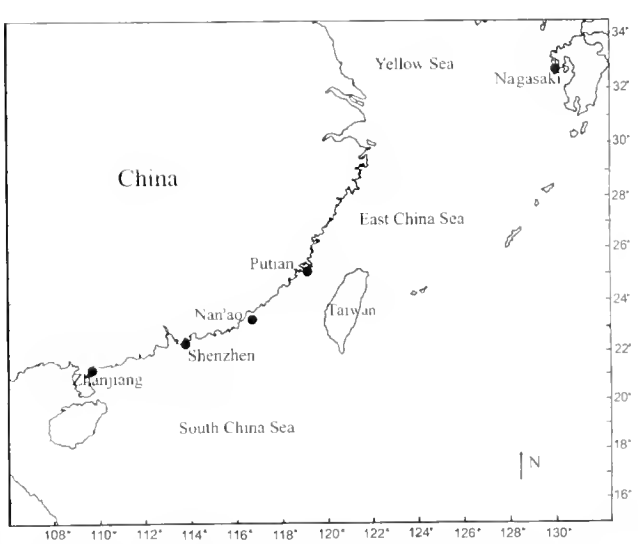

Figure 1. Sampling sites of *Sepiella maindroni* collected in the coastal waters of China and Nagasaki port in Japan.

TABLE 1.

Populations and samples used for electrophoresis and sequencing analysis in *Sepiella maindroni*

No. of Specimens				
Allozyme	Sequencing	Location	Abbreviation of Location	Date
21	6	Nagasaki	NG	1999.4.
64	5	Putian	PT	2000.4.
50	5	Nan'ao	NA	2000.5.
18	5	Shenzhen	SZ	2000.11.
49	6	Zhanjiang	ZJ	2000.10.

coding loci and 4 different kinds of tissues of *S. maindroni* (e.g. liver, eyes, mantle and buccal muscle) were screened using several enzyme-staining recipes (Shaw & Prasad 1970, Shaw & Prasad 1990, Morizot & Schmidt 1992) in a range of buffer systems.

After preliminary experiments for some specimens from five populations, 14 enzymes and optimal buffer systems and tissue (Table 3) were selected for further research.

Allele frequencies were estimated for each sample, together with several measures of genetic diversity. The proportion of polymorphic loci (P), values of the observed heterozygosity (H_o), the expected heterozygosity (H_e), and the effective number of alleles (N_a) were calculated by direct census of the population data. Two criteria of polymorphism were used: $P_{0.99}$ and $P_{0.95}$. Nei's (1972) genetic distance (D_{Nei}) was calculated to quantify the genetic divergence between cuttlefish samples. Dendrogram to illustrate the genetic relationship among five populations was constructed from genetic distance using the unweighted pair-group arithmetic mean (UPGMA) cluster-analysis algorithm (Sneath & Sokal 1973).

DNA Extraction, Amplification and Sequencing

Total genomic DNA was extracted using a CTAB method modified from Winnepenninckx et al. (1993). The target DNA segments were amplified by PCR. The primers used for the amplification of partial COI gene were: HCO2198 (5′-TAA ACT TGA GGG TGA CCA AAA AAT-3′) and LCO1491 (5′-GGT CAA CAA ATC ATA AAG ATA TTG-3′) from Folmer et al. (1994). PCR amplifications were performed under the following conditions: 120s at 94°C, then 30 cycles of 40s at 94 C, 1 min at 50°C, and 1 min at 72°C. A total volume of 25 μl. reactions consists of 0.5 units of *Taq* (TaKaRa), 0.5 μM each primer, 0.2 μM each dNTP, 2.5–3.5 mM $MgCl_2$, 2.5 μl of 10 × buffer supplied with *Taq* and 4μl (30–50 ng) of template DNA.

The amplified fragments were purified by the PCR fragment recovery Kit (TaKaRa). Purified products were sequenced directly using the ABI PRISM BigDye Terminator cycle sequencing Ready Reaction Kit and AmplicTaq DNA polymerase with ABI PRISM 377XL DNA sequencer (Applied Biosystem Inc.). All final sequences were obtained from both DNA strands for verification.

Phylogenetic and Sequence Difference Analyses

The sequences were aligned with other gene sequences (outgroup species) using a computer-assisted procedure, Clustal W (Version 1.80) (Thompson et al. 1994). No gaps were found in the COI sequences. The extent of sequence difference between individuals was calculated by averaging pair-wise comparisons of sequence difference across all individuals. One thousand bootstrap replicate data sets were produced using SEQBOOT. Under the Maximum Likelihood model, the sequence similarity and genetic distance were calculated with PHYLIP 3.5c (Felsenstein 1995). Distance-matrix trees were then constructed using the Fitch and Margoliash (1967) least-squares (LS) method and the neighbor-joining (NJ) method (Saitou & Nei 1987). *Sepia officinalis* was taken as the distant outgroup species (Carlini & Graves 1999, Accession no. AF000062).

RESULTS

Description and Interpretation of Allozymes

Of the 14 enzymes assayed routinely, 15 loci (AAT-2*, ALP*, CK*, EST-2* FBP*, G3PDH-2*, GPI-1*, GPI-2*, GRS-1*, GRS-2*, IDHP-1*, MPI-1*, MPI-2*, PGM-1*, SOD*) were monomorphic in all samples, while the others (AAT-1*, EST-1*, G3PDH-1*, GPI-3*, IDHP-2*, NP*, PGDH*, PGM-2*) showed the most common allele frequencies were less than 0.99 in at least one sample. Allozymes that were consistently and clearly scored in all samples were considered useful genetic markers for population analysis. Genetic variability was estimated by the proportion of polymorphic loci and average heterozygosity. P, the proportion of polymorphic loci ranged from 0.043 to 0.217(<0.95) and from 0.217 to 0.261 (<0.99), respectively. When five samples were

TABLE 2.

Mantle length and sex composition of *Sepiella maindroni* from five populations

No. of Specimens	Location	Mantle Length (mm)		Sex Composition	
		Range	Mean	Male	Female
21	NG	70–170	123.50	12	9
64	PT	76–258	150.6	35	31
50	NA	69.1–170	127.4	18	32
18	SZ	37–97.4	68.71	—	—
49	ZJ	33–150.3	54.97	—	—

TABLE 3.

Enzymes examined, abbreviations, E. C. number, optimal buffer systems and tissue of the study

Enzyme	E. C. No	Abbrev.	Buffer	Tissue
Aspartate aminotransferase	2.6.1.1	AAT	CAMP7.0	Mantle
Alkaline phosphatase	3.1.3.1	ALP	T-C 8.0	Liver
Creatine kinase	2.7.3.2	CK	T-C 8.0	Mantle
Esterase	3.1.1.1	EST	T-C 8.0	Liver
Glucosephosphate isomerase	5.3.1.9	GPI	CAMP7.0	Mantle
Fructose biphosphatase	3.1.3.11	FBP	T-C 8.0	Mantle
Glucose-3-phosphate dehydrogenase	1.1.1.49	G3PDH	TVB-LB8.5	Mantle
Isocitrate dehydrogenase	1.1.1.42	IDHP	CAMP7.0	Mantle
Glutathione reductase	1.6.4.2	GRS	T-C 8.0	Liver
Mannosephosphate isomerase	5.3.1.8	MPI	T-C 8.0	Eye
Nucleoside phosphorylase	2.4.2.1	NP	T-C 8.0	Eye
Phosphogluconate dehydrogenase	1.1.1.44	PGDH	CAMP7.0	Mantle
Phosphoglucomutase	5.4.2.2	PGM	CAMP7.0	Mantle
Superoxide dismutase	1.15.1.1	SOD	T-C 8.0	Liver

TABLE 4.

Allele frequencies at 8 polymorphic loci (P), sample sizes (N) and indices of genetic variability within 5 samples of *Sepiella maindroni*. H_o, mean observed heterozygosities; H_e, mean expected heterozygosity; Na, mean effective number of alleles.

		Samples				
Locus	allele	Nagasaki	Putian	Nan'ao	Shenzhen	Zhanjiang
AAT-1*	a	0.024	0.023	0.010	0.028	0.010
	b	0.976	0.969	0.970	0.944	0.929
	c	0.000	0.008	0.020	0.028	0.061
	N	21	64	50	18	49
EST-1*	a	0.024	0	0	0	0
	b	0.976	1	1	1	1
	N	21	64	50	18	49
G3PDH-1*	a	0.024	0.024	0.041	0.028	0.094
	b	0.952	0.944	0.929	0.944	0.854
	c	0.024	0.032	0.031	0.028	0.052
	N	21	62	49	18	48
GPI-3*	a	0.952	1	1	1	1
	b	0.048	0	0	0	0
	N	21	64	50	18	49
IDHP-2*	a	1	0.984	0.989	0.944	0.888
	b	0	0.016	0.011	0.056	0.112
	N	21	63	47	18	49
NP*	a	0.31	0.359	0.345	0.361	0.367
	b	0.69	0.641	0.655	0.639	0.633
	N	21	64	42	18	49
PGDH*	a	0	0.095	0.064	0.059	0.109
	b	1	0.905	0.936	0.941	0.891
	N	21	63	47	17	46
PGM-2*	a	1	1	1	1	0.99
	b	0	0	0	0	0.01
	N	21	64	50	18	49
$P_{0.95}$		0.043	0.130	0.130	0.217	0.217
$P_{0.99}$		0.217	0.217	0.217	0.217	0.261
H_o(S.E)		0.031 (0.008)	0.034 (0.009)	0.034 (0.010)	0.041 (0.012)	0.050 (0.013)
H_e(S.E)		0.031 (0.008)	0.036 (0.010)	0.034 (0.010)	0.039 (0.010)	0.055 (0.014)
Na		1.212	1.235	1.240	1.302	1.294

For 5 samples pooled: $P_{0.95} = 0.147$; $P_{0.99} = 0.226$

Mean heterozygosities per locus = 0.038(H_o); 0.039(H_e)

The average effective number of alleles = 1.26

TABLE 5.

Genetic similarity (I) and genetic distance (D_{Nei}) among five samples in the cuttlefish *Sepiella maindroni*

Samples	Nagasaki	Putian	Nan'ao	Shenzhen	Zhanjiang
Nagasaki		0.9993	0.9996	0.9994	0.9982
Putian	0.0007		0.9999	0.9998	0.9992
Nan'ao	0.0004	0.0001		0.9999	0.9992
ShenZhen	0.0006	0.0002	0.0001		0.9994
Zhanjiang	0.0018	0.0008	0.0008	0.0006	

pooled, $P_{0.95} = 0.147$; $P_{0.99} = 0.226$. Mean heterozygosities per locus (observed and expected), and the effective number of alleles per locus were presented for all of the five populations pooled (Table 4).

Genetic similarity (I) and genetic distance (D_{Nei}) between all samples based on the 23 loci were given in Table 5. Nei's D of *S.maindroni* populations varied from 0.0001 to 0.0018 (Table 5).

Comparison of observed genotypic frequencies with Hardy-Weinberg expectation did not show significant deviation ($P > 0.05$) at NP* loci of five populations. The expected values of the genotype frequency were smaller than five in other polymorphic loci, so a χ^2 test for the Hardy-Weinberg equilibrium could not be performed. The dendrogram was constructed from Nei's D value (Fig. 2.)

Sequence Variation of the COI Gene

A base-pair (bp) (661) segment of the COI gene was sequenced for 27 individuals from five locations (NG, PT, NA, SZ and ZJ). Target segments were given by base pairing.

The A + T percentage of 5 samples ranged from 68.38% to 68.99% (Table 6), which is a little higher than *S. officinalis*'s (63.47%) (Carlini & Graves 1999)

Among these individuals, eleven nucleotide positions (position 146, 197, 263, 317, 362, 497, 543 and 617) were found to be variable (Fig. 3). As expected, ten variation sites were at third position of codon triplets, and only one variation site (position 621) was at 1st position of codon triplets (Fig. 3). The base of positions 621 and 623 occurred to transverse from A to T at the same time in four individuals checked, and finally resulted in compatible amino acid change, in which Cys was in place of Ser. The other base substitutions were silent (synonymous) changes that do not result in amino acid change.

Analysis of Relationships and Occurrence Frequency of Haplotypes

Nucleotide sequence difference between 27 individuals from Nagasaki in Japan to Zhanjiang in South China Sea ranged from 0.00 to 1.21%. Among these individuals, nine unique haplotypes (A–I) were found (Table 7). The haplotype D, with a frequency of 48% (13/27), was most commonly observed in all samples, though it was found only in the 4 populations living in the coastal waters of China. The haplotype A followed, and its highest frequency (4/6) was found in NG. The haplotype E was found in PT and ZJ. The other haplotypes were found each in only one individual.

According to the phylogenetic tree of Neighbor-Joining (Fig. 4) and UPGMA (Fig. 5), there were two main branches: most of NG (67–83%) occupied one position from one branch, and the individuals (81–86%) living in the coastal waters of China took up the other branch. But genetic distance of 27 individuals as revealed by

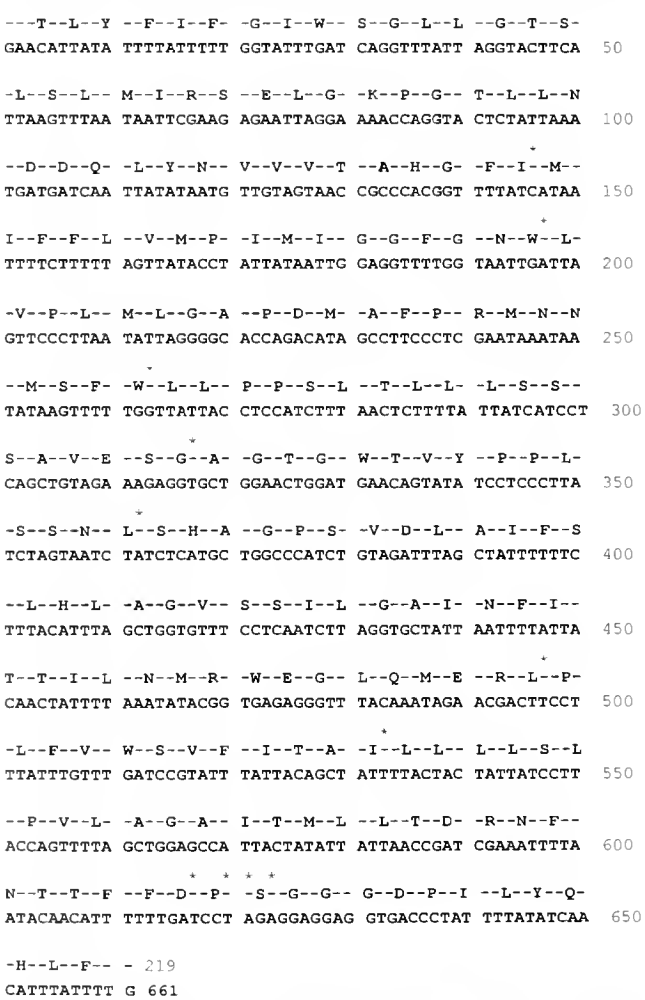

Figure 3. Nucleotide and deduced amino acid sequences of partial COI gene fragment in *S. maindroni*. Asterisks above the sequence show the variation nucleotide positions.

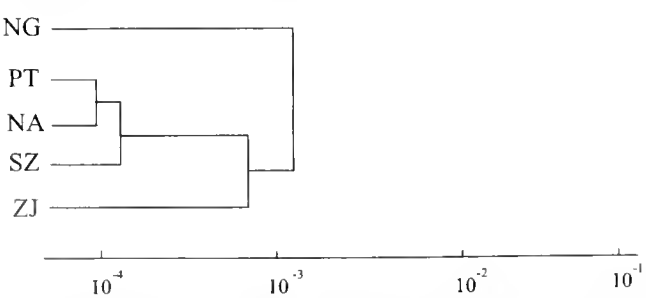

Figure 2. Dendrogram derived from Nei's genetic distance (Nei,1972) using UPGMA cluster analysis.

TABLE 6.

Comparison in base composition of COI gene

Sepiella Maindroni	A%	C%	G%	T%	Accession No.
Nagasaki	0.286	0.159	0.151	0.404	AF346853
Putian	0.283	0.163	0.153	0.401	AF340032
Nan'ao	0.284	0.163	0.153	0.399	AF361360
ShenZhen	0.284	0.159	0.153	0.404	AF361359
Zhanjiang	0.280	0.163	0.153	0.404	AF361361
Outgroup					
Sepia officinalis	0.265	0.192	0.174	0.370	AF000062

A: adenine; C: cytosine; G: guanine; T: thymine

the COI sequence ranged from 0.0000 to 0.0076 using PHYLIP 3.5c. Geographical proximity had no remarkable relevance to genetic-phylogenetic relationships among these haplotypes.

DISCUSSION

The study of enzyme loci in five populations of *S. maindroni* showed that this species had low levels of variability. The mean H_o(0.038) falls below the average for invertebrate and molluscs (0.120 and 0.145, respectively) (Ward et al. 1992), and also for marine molluscs (0.147) (Fujio 1997), but within the range for cephalopods (0.03 ± 0.03) (Zheng et al. 2001a). Low levels of heterozygosity have been found in other species of Cephalopoda, such as *Loligo gahi, Illex argentinus* (Carvalho & Pitcher 1989, Carvalho et al. 1992), *Sepioteuthis lessoniana* (Izuka et al. 1994), *Sepia officinalis, S.orbignyana, S.elegans* (Perez-Losada et al. 1996). Very low levels of genetic variability are apparently a common facet of cephalopods except *Berryteuthis magister* (H_o = 0.131) (Katugin 1993), a feature probably unique over any major group of invertebrates.

In the five populations, the proportion of polymorphic loci of NG was the lowest ($P_{0.95}$ = 0.043), and ZT was the highest. The sample of NG was obviously different from other samples at loci of AAT-1*, EST-1*, GPI-3*, IDHP-2* and PGDH*. The fixed allelic differences at AAT-1* between NG and other samples in coastal waters of China could serve as valuable markers to monitor geographic variation in stock distribution, though the genetic distance was low (maximum value of D = 0.0018 being found between the NG and ZJ samples).

Populations undergoing severe bottlenecks with rapid reductions in population size may lose rare alleles through genetic drift (Wright 1978). Severe fishing pressure could bring about such population declines, especially if populations were founded each year by relatively few individuals. Population crashes are more likely in commercially harvested species (Nelson & Soule 1987), particularly when production models over-estimate escapement rates. In annual species such as *S. maindroni*, the genetic implications of bottlenecks are particularly significant since there are no overlapping year classes to serve as a repository for genetic diversity. For instance, in China, the cuttlefish *S. maindroni* had once become one of the four biggest fisheries in the East China Sea. But now, its position has been replaced by other cephalopods. Clearly, it is facing the danger of extinction. A bottleneck effect may be one of the reasons for the low diversity of *S. maindroni* populations.

The present COI gene analysis suggests that there was no remarkable genetic differentiation in the five samples, though there was evidence to indicate that NG samples differ slightly from the other samples in the coastal waters of China, especially in the number and type of haplotypes and the position in UPGMA and NJ trees. The result also supported the conclusion of biochemical research.

Pelagic organisms in the open ocean were generally regarded as having low levels of population differentiation, resulting from probably ample opportunities of dispersal in various life history stages and the lack of physical barriers in the environment. The study on the population genetic structure of large pelagic fishes and some cephalopods in the open oceans, such as swordfish and

TABLE 7.

Variable nucleotide positions in part of the COI gene region of 11 haplotypes, and number of individual of each haplotype found in each locality.

	Site											Number of Individuals in Each Locality					
Haplotype	146	197	263	317	362	497	543	617	620	621	623	NG	PT	NA	SZ	ZJ	Total
A	C	A	G	T	A	T	T	T	T	A	A	4	0	0	1	0	5
B		G	·	·	·	·	C	·	·	·	·	1	0	0	0	0	1
C	·	G	·	·	·	·	C	C	·	·	·	1	0	0	0	0	1
D	·	G	·	·	T	·	C	C	·	·	·	0	3	4	3	3	13
E	·	G	·	·	T	·	C	C	·	T	T	0	1	0	0	2	3
F	·	G	·	·	T	·	C	C	A	T	T	0	1	0	0	0	1
G	·	G	·	C	·	C	C	·	·	·	·	0	0	1	0	0	1
H	·	G	·	·	·	·	·	·	·	·	·	0	0	0	1	0	1
I	T	G	A	G	·	·	C	·	·	·	·	0	0	0	0	1	1

Dots(·) indicate identities.

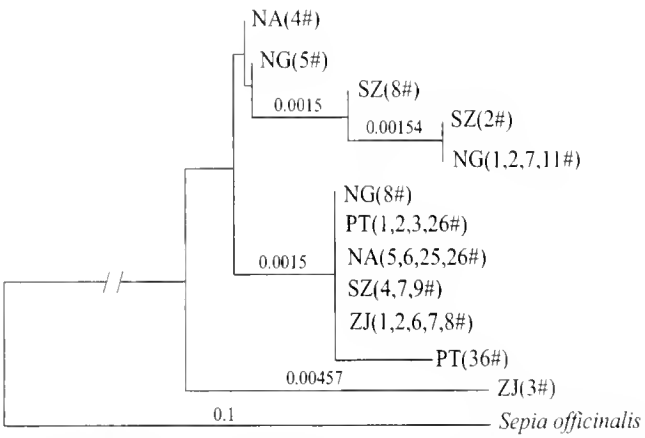

Figure 4. Neighbor-Joining tree based on COI gene sequences. *Sepia officinalis* was considered as distant outgroup species.

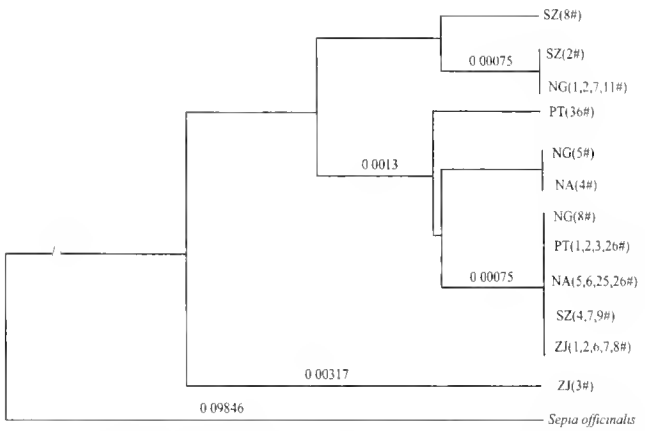

Figure 5. UPGMA phylogenetic tree based on COI gene sequences. *Sepia officinalis* was used as the distant outgroup species.

diamond-shaped squid, showed very low levels of genetic diversity (Rosel & Block 1996, Kitaura et al. 1998).

The cuttlefish *S. maindroni* is distributed widely in Asia—in the sea of Honshu (Japan) to the north, Malaysia and the Philippine Islands to the south, and Indian Ocean to the west. The present study suggests that for *S. maindroni*, much like a pelagic species with considerable mobility, behaves so that all the populations covered in this study may have migrated into each other and no physical barriers existed in the environment, especially regarding the samples from PT to ZJ.

Genetic diversity in populations of the cuttlefish needs to be further examined with more extensive data from different geographical populations and also from more DNA markers such as highly variable microsatellite DNA markers.

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SEASONAL VARIATION IN PHYSIOLOGICAL CONDITION OF *AMBLEMA PLICATA* IN THE UPPER MISSISSIPPI RIVER

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ABSTRACT Measures of physiological condition are being used as sub-lethal endpoints in studies with unionids exposed to a variety of stressors, yet the natural seasonal variation in these measures are largely undocumented. We measured concentrations of glycogen in foot and mantle tissue and a tissue condition index (TCI) in *Amblema plicata* (Say 1817), about monthly, for 2 years in mussels that were: (1) obtained directly from the Upper Mississippi River (riverine group); and (2) relocated from the river into an artificial pond (relocated group). In both groups, we observed significant seasonal variation in all physiological indicators. Seasonal variation in glycogen was 72% in mantle and 52% in foot tissue and paralleled reproductive activity in this short-term breeder. In the relocated group, most of the variation in glycogen occurred during the first six months after relocation, suggesting that handling stress may have been a contributing factor. The significant seasonal variation in the TCI paralleled glycogen in riverine mussels. We observed tissue-specific differences in glycogen in the riverine group, but not in the relocated group. These data suggest that an interaction of environmental and biological factors influence the energetic status of mussels in natural populations. A better understanding of this variation is needed to interpret changes in physiological condition due to stressors such as relocation.

KEY WORDS: glycogen, condition index, seasonal variation, *Amblema plicata*, relocation

INTRODUCTION

Unionids are the most imperiled group of animals in North America with 72% of the 297 species classified as extinct, endangered, threatened, or listed as a species of special concern (Williams et al. 1993). Declines in species density and diversity have been attributed to habitat degradation, changes in fish host distributions, commercial exploitation, and introduced species (Bogan 1993). Thus, many agencies are examining approaches to conserve this fauna. Relocation is being used extensively as both a conservation and management tool (Cope & Waller 1995), however, it is difficult to draw conclusions on the relative success of relocations because of inconsistencies in the methods used and in the selection of endpoints. Often, survival and growth are the only endpoints measured and they are usually monitored for <1 yr (Cope & Waller 1995). However, one-year survival estimates may underestimate the mortality that occurs during longer relocations (Newton et al. 2001), and short-term measures of growth may be of limited value for these long-lived animals (Naimo et al. 1998).

Research suggests that changes in certain physiological measures often precede changes in survival (Haag et al. 1993). However, Newton et al. (2001) were unable to predict mortality in *Amblema plicata* based on physiological indicators measured annually over a three- to four-year period. This lack of predictive power may have been related to the frequency of measurement or because the measures were not sensitive enough. It is difficult to draw conclusions about the physiological condition of unionids from measurements made at a few points in time without understanding the seasonal variation in such measures.

Various sub-lethal indicators have been examined in unionids. TCI has been used to assess the condition of freshwater bivalves exposed to zebra mussels (Baker & Hornbach 1997) and contaminants (Naimo et al. 1990), or infected with parasites (Jokela et al. 1993). Because the measurement of condition indices requires sacrificing the individual, their application is limited. More recent techniques allow tissues to be biopsied for physiological and biochemical constituents (Berg et al. 1995, Byrne & Vesk 1996, Naimo et al. 1998). Glycogen is the primary storage form of carbohydrates in bivalves; consequently, it is being used as a physiological indicator in unionids subjected to emersion stress (Greseth 1998), quarantine and relocation (Patterson et al. 1997, Naimo et al. 1998), zebra mussel infestation (Haag et al. 1993, Hallac & Marsden 2000) and parasitic infestations (Jokela et al. 1993).

The few studies measuring seasonal variation in physiological indicators in natural populations suggest that much of this variation is related to environmental conditions or reproduction (Dietz & Stern 1977, Holopainen 1987, Pekkarinen 1993). For example, monthly sampling of *Sphaerium transversum* for total protein and carbohydrates showed a positive correlation with reproductive activity during the fall and winter and a decline towards the end of the reproductive season (Dietz & Stern 1977). Pekkarinen (1993) found the periods of gravidity for *Unio pictorum* and *U. tumidus* were correlated with rising and maximum water temperatures and condition indices rose concomitant with the rise in water temperatures. Seasonal changes in the physiological condition of unionids is a complex process involving, at a minimum, environmental conditions and the energetic demands of reproduction.

Most studies have measured physiological condition at one point in time in response to a given stressor. In addition, most of these studies measured seasonal variation in physiological indicators on bivalves with substantially different reproductive cycles than unionids. Information on the seasonal variability in physiological measures in individuals is largely undocumented. Thus, our objectives were to (1) examine the seasonal variation in glycogen concentrations and a TCI in two groups of *A. plicata*, an undisturbed riverine group and a relocated group; and (2) compare seasonal patterns in glycogen between foot and mantle tissue.

MATERIALS AND METHODS

We examined the seasonal variation in glycogen concentrations and in a TCI in two groups of *A. plicata*: a riverine group and a relocated group. For the riverine group, we obtained 10 zebra-

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mussel-free individuals (80–90 mm shell length) about monthly from May 1997 to October 1998 from Lake Onalaska, a riverine lake in the Upper Mississippi River, near La Crosse, WI. For the relocated group, we relocated 200 zebra-mussel-free individuals (80–90 mm shell length) from the Upper Mississippi River into an 0.04 ha earthen pond supplied with well-water at the Upper Midwest Environmental Sciences Center in La Crosse, WI, in May 1995. Ten individuals were obtained from the pond for analysis about monthly until November 1996. Although no supplemental food was added, the pond produced extensive blooms of green algae each summer. Because these two groups were sampled during different years, we have made no attempt to compare results between groups; rather, we are examining seasonal patterns in physiological measures within a group and between tissues within a group.

To obtain tissues for analysis, 50–100 mg pieces of foot and mantle tissue were removed from sacrificed animals, placed on dry ice, and stored at −84°C until analyzed for glycogen. Tissue samples were consistently removed from the same area (Naimo & Monroe 1999). The remainder of the soft tissues and shells were dried to a constant weight at 105°C for determination of the TCI, calculated by dividing the tissue dry mass by the shell dry mass and multiplying the quotient by 100. For the relocated group, TCI analysis was conducted only in 1996. Because *A. plicata* are not sexually dimorphic, the sex of individuals was not determined. Concentrations of glycogen were determined by the alkaline digestion and phenol-sulfuric acid spectrophotometric method (Naimo et al. 1998).

The accuracy of glycogen determinations was quantified by the use of procedural blanks, replicates of an in-house reference material (Naimo et al. 1998), triplicate analysis of 4 aqueous calibration standards, and triplicate analyses of 3 known additions or 3 matrix standards. Glycogen in the tissue samples averaged 0.17 mg (range, 0.03–0.97) in foot tissue and 0.29 mg (range, 0.04–1.86) in mantle tissue. As a measure of precision, we estimated the relative standard deviation of triplicate analyses of known additions according to APHA et al. (1995). Relative standard deviations averaged 8% in foot tissue and 12% in mantle tissue. Bias associated with our glycogen determinations was estimated by recovery of matrix standards and known additions (APHA et al. 1995), mean percent recovery of glycogen averaged 106% in foot tissue and 99% in mantle tissue.

We assumed glycogen concentrations (mg/g dry weight) followed a gamma distribution. The gamma distribution is a plausible model for random variables, like glycogen concentration, that cannot take values less than zero and for which the variance is approximately proportional to the squared mean (McCullagh & Nelder 1989). We used the model for mean glycogen concentration in tissue j from group i, μ_{ij}, given by:

$$\mu_{ij} = \mu_0 \exp\left[\tau_j + \beta \log(t) + \beta_j \log(t) + \gamma_1 \sin\left(\frac{2\pi t}{12}\right) + \gamma_2 \cos\left(\frac{2\pi t}{12}\right)\right], \quad (1)$$

where μ_0 is the baseline (mantle tissue from mussels in the pond) mean glycogen concentration, τ_j is the effect of tissue type (foot or mantle), t is time measured in months since the start of the year during which the trial began, β is a trend coefficient, β_j is a trend coefficient due to tissue j and γ_1 and γ_2 are coefficients in a sinusoidal seasonal effect at a frequency of one cycle per year. This model allows us to detect long-term trends in glycogen, including seasonal effects that can be described by a sinusoidal response, and possible differences between tissues in each group. We modeled Var(μ) by:

$$Var(\mu) = \frac{\mu^2}{\phi}, \quad (2)$$

where ϕ is a scale parameter. Log(μ) can be viewed as a generalization of analysis of covariance that accommodates a gamma-distributed response, one categorical main effect (tissue), a sinusoidal seasonal effect, and a logarithmic trend that may differ between tissues. We fitted the model given by equations (1) and (2) using maximum likelihood estimation. We modeled the mean TCI similarly to the model given above for glycogen, except the parameters for tissue type were removed.

RESULTS

In the riverine group, glycogen and the TCI varied significantly on a seasonal basis (glycogen $P = 0.02$, TCI $P < 0.0001$), and showed a similar pattern in both years with highest values early in the season (usually June) and lowest values late in the season (usually October; Fig. 1). Although a significant seasonal pattern was apparent in all indicators, it was most pronounced in glycogen in mantle tissue. For example, glycogen concentrations varied 72% [(annual maximum − annual minimum) / annual maximum) × 100], during 1997 and 46% during 1998 (Fig. 1). In contrast, the seasonal change in TCI varied only 20% during 1997 and 38% during 1998. Although seasonal patterns in glycogen and TCI were similar in both years, the annual maximum of glycogen in foot and mantle tissue was greater in 1997 than in 1998, while TCI maximums were similar between years.

In the relocated group, glycogen and the TCI also varied seasonally (glycogen $P = 0.01$, TCI $P = 0.002$). Like the riverine group, highest values were observed early in the season (usually May) with lower values each fall (usually November). Most of the seasonal variation in physiological measures in this group were likely driven by the large decline in glycogen the first 6 months after the relocation (Fig. 1). Glycogen values declined by 72% in mantle tissue and 65% in foot tissue from May to November 1995. During 1996, glycogen concentrations continued to decline, but only by 35% in mantle tissue and 49% in foot tissue.

Glycogen concentrations varied between foot and mantle tissue in the riverine mussels ($P < 0.0001$), but not in the relocated mussels ($P = 0.09$). In the riverine group, glycogen concentrations in mantle tissue ranged from 1.9 to 3.6 times those in foot tissue over both years. In the relocated mussels, glycogen concentrations in foot and mantle tissue were similar during both years (Fig. 1).

DISCUSSION

The significant seasonal variation in glycogen was anticipated, given that glycogen is the primary reserve energy store and is re-allocated from the body towards the gonads during gametogenesis (Gabbott 1983). It is difficult to compare the amount of annual variation in glycogen concentrations in our riverine group (range, 32–72%) with other studies because few have measured glycogen with the frequency that we used and several have used species with substantially different reproductive traits. However,

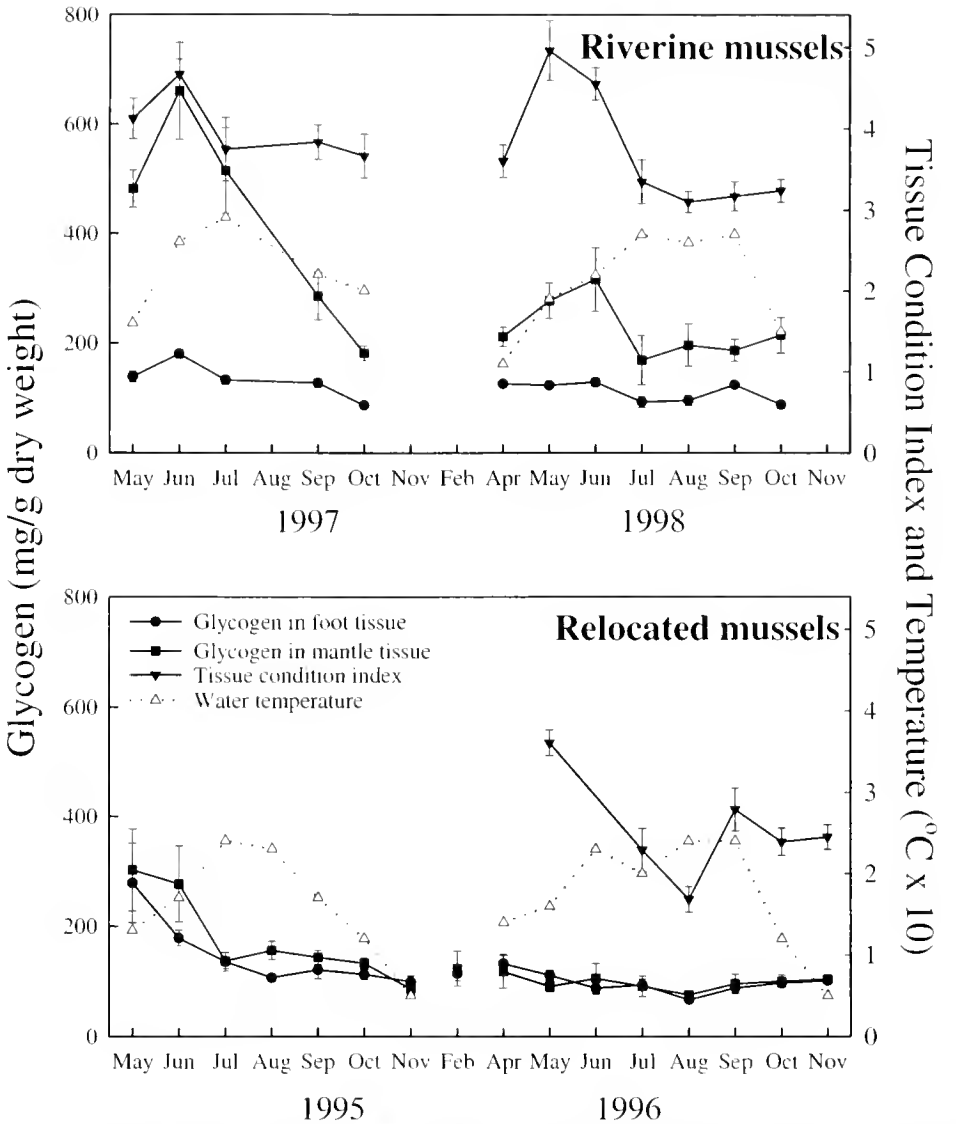

Figure 1. Mean (±1 SE) concentrations of glycogen and the tissue condition index in *Amblema plicata* (solid lines) and mean water temperature (dotted lines). Riverine mussels were sampled from the Upper Mississippi River about monthly from May 1997 to October 1998. Relocated mussels were removed from the Upper Mississippi River and placed into an earthen pond (May 1995), then sampled about monthly until November 1996.

total body glycogen content varied 39% between July and October in *Anodonta piscinalis*, during the time of glochidial development (Jokela et al. 1993). Similarly, the total carbohydrate content of fingernail clams (*S. transversum*) varied 80% when sampled monthly in a given year (Dietz & Stern 1997). Thus, it seems that significant seasonal variation exists in glycogen concentrations in a variety of freshwater bivalves, even those with substantially different reproductive strategies.

Seasonal variation in glycogen concentration is likely driven by environmental factors such as water temperature, which subsequently influences reproductive condition. *A. plicata* is a short-term breeder with maturing gametes and glochidia present from May to early August in the Upper Mississippi River (Holland-Bartels & Kammer 1989). By late May, 100% of the females and 80% of the males contain mature gametes, indicating a progression towards full reproductive maturation as water temperatures rise from 13° to 19°C (Fig. 2). Seasonal patterns in glycogen concentration in riverine mussels in this study parallel reproductive activity. As water temperatures warm in the early spring, male and female mussels begin producing mature gametes and glycogen is either being stored or is re-allocated from other tissues to meet the energetic demands of reproduction. As temperatures begin to stabilize in late June, gametes are fertilized, mature glochidia are released, and glycogen concentrations are depleted (Fig. 2).

Condition indices are frequently used as a measure of nutritive status in marine bivalves and represent the total energetic status of an individual, including stores of protein, lipids, and carbohydrates (Bayne et al. 1985). Thus, seasonal patterns in condition indices should follow food availability and reproductive status. In the riverine mussels in this study, seasonal patterns in the TCI paralleled reproductive activity. Prior to reproduction (April to June), the condition of mussels increased (they were adding soft tissue

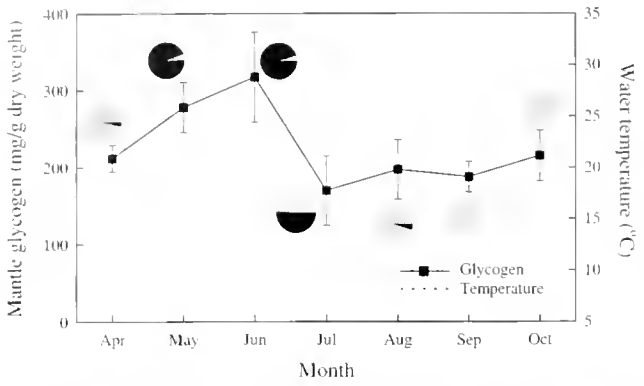

Figure 2. Mean (±1 SE) concentrations of glycogen in mantle tissue in *Amblema plicata* from the Upper Mississippi River during 1998 (solid line), and water temperature at the time of mussel collection (dotted line). Pie charts estimate the reproductive stage in *A. plicata* with the black area representing the percentage of male and female mussels on each date containing mature gametes (data from Holland-Bartels & Kammer 1989).

weight) as they prepared for reproduction. After fertilization and glochidia release (June to July), energetic stores were depleted and the TCI decreased. This coupling of the condition index and the reproductive cycle was also seen in the short-term breeders *U. tumidus* and *U. pictorum* (Pekkarinen 1993).

The similarity in the seasonal patterns between glycogen and the TCI in riverine *A. plicata* suggests that in natural populations, glycogen represents a large proportion of the total energy stored. In another short-term breeder, *Quadrula pustulosa*, carbohydrates averaged 76% of the total energy stores (Greseth 1998). Conversely, in relocated mussels, patterns in glycogen and the TCI become uncoupled, suggesting that energetic stores besides glycogen are being used. In natural populations, we hypothesize that measures of glycogen and the TCI provide comparable data on the relative condition of an individual, but when unionids are stressed, such as during relocation, these two measures provide different data, reflecting varying energy sources during periods of stress. If this can be shown for additional species, resource managers may have another tool for use in studies with threatened and endangered species.

The substantial difference in glycogen between tissues in riverine mussels, but not in relocated mussels, suggests that energy stores may be re-allocated during periods of stress. These data support our earlier conclusions that glycogen may be preferentially stored in mantle tissue in *A. plicata* when environmental conditions are favorable (Naimo & Monroe 1999). Jokela (1996) found that energy was allocated differently among reproduction, somatic growth, and biochemical storage, depending on the time of the year when *A. piscinalis* were transplanted. Thus, because the relocated mussels were moved during an active reproductive period, the glycogen that had accumulated in mantle tissue may have been re-allocated to maintenance metabolism.

The seasonal variation in physiological measures in native populations indicates that numerous biological and environmental parameters interact to influence the relative condition of unionids. Researchers measuring physiological responses of unionids to a particular stressor (zebra mussels, contaminants, relocation, etc.) at a given time need to understand where these measures fall on the natural response curves to better interpret how a given stressor affects the physiological energetics of the species under consideration. Finally, this research was conducted on a single species; studies with other species are critically needed before resource managers can make sound management decisions that will conserve this declining faunal group.

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EFFECTS OF STOCKING DENSITY ON GROWTH AND SURVIVAL OF ATLANTIC SURFCLAMS IN BOTTOM CAGES VERSUS MESH BAGS

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ABSTRACT Atlantic surfclams, *Spisula solidissima*, were reared in field culture to determine the effects of: (a) stocking densities on clams cultured in bottom cages and mesh bags; (b) bottom cage versus mesh bag culture; and (c) bag mesh diameter on growth and survival of clams in coastal Georgia. With the exception of the 100 stocking density, mean clam size decreased with increases in stocking densities in cages, i.e., 200 clams/m^2 ($\bar{x}$ = 56.5 mm) = 300 ($\bar{x}$ = 57.0 mm) > 100 ($\bar{x}$ = 52.8 mm) > 400 ($\bar{x}$ = 49.1 mm) = 500 ($\bar{x}$ = 51.0 mm) > 600 ($\bar{x}$ = 46.5 mm). No significant difference ($p = 0.1994$) in clam survival occurred among any cage treatments; however, mean survival of clams ranged from 52% in cages stocked at 300 clams/m^2 to 87% in cages stocked at 500 clams/m^2. Significant differences ($p = 0.0258$) in clam survival occurred among the mesh bag stocking density treatments with survival ranging from 22% in densities of 100 clams/m^2 to 73.5% at 500 clams/m^2. In the mesh bags, clam size was significantly greater in the 200 clams/m^2 ($\bar{x}$ = 32.9 mm) = 400 clams/m^2 ($\bar{x}$ = 32.8 mm) = 500 clams/m^2 ($\bar{x}$ = 34.6 mm) treatments than 300 clams/m^2 ($\bar{x}$ = 29.4 mm) which were greater than the 100 clams/m^2 ($\bar{x}$ = 26.6 mm) bag treatment. Atlantic surfclams had significantly ($p = 0.0100$) higher survival in 3-mm mesh (62%) versus 6-mm mesh bags (18.5%) with low survival (0.3%) in 12-mm mesh bags. Clam size was similar ($p = 0.0623$) in all three mesh size treatments. Atlantic surfclams had greater growth and survival rates with greater production in bottom cages over mesh bags for the field culture of clams in Georgia. A culture strategy using stocking density data for maximizing the harvest, hence marketing window of the Atlantic surfclam, is discussed.

KEY WORDS: *Spisula*, growth, mortality, stocking density, cage, bag, mesh

INTRODUCTION

The Atlantic surfclam, *Spisula solidissima* (Dillwyn, 1817), natural fishery was valued at $30.4 million in the United States in 1999 (O'Bannon 2000). Atlantic surfclams occur from shore to 40 m depth offshore, from the maritime provinces in Canada to South Carolina (Abbott 1974). However, the fishery is centered primarily between New York and Virginia. Large adult clams greater than 12 cm in shell length (L) are harvested and used in the fried clam strip and chowder markets (Goldberg 1989).

The Atlantic surfclam does not naturally occur in Georgia; however, a subspecies *Spisula solidissima similis* does occur (Walker & Heffernan 1994). The southern surfclam, *Spisula solidissima similis* is now classified as *Spisula raveneli* (Conrad, 1831) (American Fisheries Society 1998). Insufficient stocks of *Spisula raveneli* occur in coastal Georgia to support a fishery (Walker & Heffernan 1994). The Atlantic surfclam cannot survive the summer water temperatures in coastal Georgia, whereas the southern surfclam survives the first summer, but generally dies during the second summer (Walker & Heffernan 1994).

Research into the development of an Atlantic surfclam aquaculture fishery in Georgia has shown promise (Goldberg & Walker 1990; Walker & Heffernan 1990a, Walker & Heffernan 1990b, Walker & Heffernan 1990c). Earlier growth studies examining the feasibility of culturing the northern Atlantic surfclam in Georgia showed that surfclams grow well over winter, but die by summer (Goldberg & Walker 1990, Walker & Heffernan 1990a, Walker & Heffernan 1990b, Walker & Heffernan 1990c). Atlantic surfclams (10 mm in L) when field planted in October after water temperatures drop below lethal levels (28°C) in Georgia will grow 50 to 70 mm L by the following spring. Thus, marketable size Atlantic surfclams can be grown in 6 months as compared to 2 to 2.5 years of growth required for culturing the native northern quahog, *Mercenaria mercenaria* (Linnaeus, 1758) (Walker 1997).

The potential for aquaculture for Atlantic surfclams is to produce clams (approximately 50 mm L) for the lucrative fried, raw and steamer markets. The rapid growth rate of the clam in Georgia allows a marketable product in six months from field planting. Extension efforts in developing an aquaculture industry for surfclams has also shown promise. Taste tests at local seafood festivals in Georgia indicated that the clam has potential for development into an aquaculture industry (unpublished data). Taste testers rated the clam as an excellent product with a slight sweet and mildly nutty flavor; however, clams must be placed in floating cages for a brief period to allow clams to purge sand from inside their shell prior to marketing. Marketing attempts with local seafood dealers showed that they could sell the clam at an equivalent price to that of the northern quahog. The only drawback of the marketing attempts was that the Atlantic surfclam had a short shelf life. A preliminary attempt at flash freezing the clams and placing them in shrink packing, resulted in the cracking of the shells. The shells were weak presumably due to the rapid growth rate of clam. Clams were increasing their length and width at the margins of the shell rather than producing a thick layer of shell. Another problem in developing an aquacultural industry for surfclams in Georgia is that clams need to be harvested, sold, and consumed in a very short period (May). One possible means of increasing the marketing window and increasing shelf life, may be to harvest clams earlier in the season prior to the warmer waters adding an increased physiological stress level on the animal. By manipulating field stocking densities of surfclams in culture units, a clam farmer may optimize growth conditions producing a harvestable product earlier in the season.

This study examines the effects of cage and mesh-bag stocking densities on the growth and survival of cultured surfclams in the coastal waters of Georgia in an effort to optimizing the field harvesting window of opportunity. In addition, the study determines

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the effects of bag mesh size on growth and survival of the Atlantic surfclam.

MATERIALS AND METHODS

Cage Stocking Density Study

To determine the effects of cage stocking density on growth and survival of Atlantic surfclams, cages were stocked with Atlantic surfclams of 15.5 ± 0.17 (S.E.) mm L at treatment densities of 100, 200, 300, 400, 500, and 600 clams per cage. Each treatment had three replicate cages. Eighteen 6-mm mesh vinyl-coated-wire cages (1 × 1 × 0.6 m) were partially buried (0.3 m) in a sandy-mud substrate in a straight line at the spring-low-water mark on an intertidal flat at the mouth of House Creek, Little Tybee Island, Georgia. Two 1-m poles (to anchor cages) were driven into the sediment at opposite corners of each cage and attached to the cage with cable ties. Cages were randomly stocked according to a random number table in October 5, 1995. Cages were harvested 222 days later in May 14, 1996 with all clams per cage counted and 50 randomly selected clams measured for shell length (anterior-posterior) to the nearest 0.5 mm with Vernier calipers..

To determine the effects of stocking density on growth utilizing the mesh-bag-line culture system designed for the culture of the northern quahog (Walker & Hurley 1995), surfclams were stocked at various densities in 6-mm mesh bags. Five mesh bags (0.5 m^2 each) were attached to a long line according to Walker and Hurley (1995). Atlantic surfclams at 15.5 ± 0.17 mm were stocked at densities of 50, 100, 150, 200, and 250 clams per bag with three replicate bags per treatment. These stocking densities are equivalent to densities of 100, 200, 300, 400, and 500 clams/m^2, respectively. Results are reported in terms of clams/m^2 to compare with the same density as in the cage experiment. Bags were randomly assigned to long lines and were deployed in October 5, 1995. Bag lines were secured with stakes adjacent to the cages. Clams were harvested in May 14, 1996 with all clams per bag counted and 50, if available, randomly selected clams measured for shell length (anterior-posterior) to the nearest 0.5 mm with Vernier calipers.

Mesh Size Study

To determine the effects of bag mesh diameter on growth and survival of Atlantic surfclams, 3-mm (N = 4), 6-mm (N = 3), and 12-mm (N = 3) mesh bags were stocked with 200 Atlantic surfclams at a mean size of 15.5 ± 0.17 mm. Five bags each were randomly attached to two long lines (Walker & Hurley 1995) and staked at 0.5 m below the spring-low-water mark on an intertidal flat at the mouth of House Creek, Little Tybee Island, Georgia. Bags were harvested in May 14, 1996 with all clams per bag counted and 50 individuals measured for shell length.

Statistical Analysis

One-way Analysis of Variance (ANOVA) ($\alpha = 0.05$) and Tukey's Studentized Range Test (SRT) ($\alpha = 0.05$) using SAS for PC (SAS Institute Inc. 1989) were carried out in each study. All proportion survival data was arcsine square root transformed prior to statistical analysis.

RESULTS

Cage and Mesh Bag Stocking Density Study

Greater Atlantic surfclam survival ($p = 0.0040$) occurred in cages (77%) than in mesh bags (53%). No significant difference ($p = 0.1994$) in clam survival occurred among any cage treatments (Table 1); however, mean survival of clams ranged from 52% in cages stocked at 300 clams/m^2 to 87% in cages stocked at 500 clams/m^2. Significant differences ($p = 0.0258$) in clam survival occurred among the mesh bag treatments with survival ranging from 22% in densities of 100 to 73.5% at 500 clams/m^2. Although survival of clams in bags increased with increases in stocking densities (Table 1), clam survival at the 100 clams/m^2 stocking density was only significantly lower than that at 500 clams/m^2.

Significant differences in clam size occurred among stocking densities in both cages ($p < 0.0001$) and mesh bags ($p < 0.0001$; Table 1). In general and with the exception of 100 stocking density, mean clam size decreased with increases in stocking densities in cages, i.e., 200 clams/m^2 ($\bar{x}$ = 56.5 mm) = 300 clams/m^2 ($\bar{x}$ = 57.0 mm) > 100 clams/m^2 ($\bar{x}$ = 52.8 mm) > 400 clams/m^2 ($\bar{x}$ = 49.1 mm) = 500 clams/m^2 ($\bar{x}$ = 51.0 mm) > 600 clams/m^2 ($\bar{x}$ = 46.5 mm). In the mesh bags, clam size was significantly lower in the 100 clams/m^2 bags ($\bar{x}$ = 26.6 mm) which was greater than 300 ($\bar{x}$ = 29.4 mm) > 200 clams/m^2 ($\bar{x}$ = 32.9 mm) = 400 clams/m^2 ($\bar{x}$ = 32.8 mm) = 500 clams/m^2 ($\bar{x}$ = 34.6 mm). Overall in the mesh bags, clam size increased with increases in stocking density. Surfclams achieved a greater size in cages versus mesh bags regardless of stocking density. In cages, mean Atlantic surfclam size ranged from 46.5 mm at 600 clams/m^2 to 57 mm at 300 clams/m^2, whereas, in mesh bags mean clam size ranged from 26.6 mm in 100 clams/m^2 to 34.6 mm in 500 clams/m^2 treatments (Table 1).

Mesh Size Study

Growth and survival data of the Atlantic surfclam cultured in various mesh bag diameters are given in Table 2. ANOVA showed that no significant difference ($p = 0.0623$) in clam size occurred among treatments; however, Tukey's SRT showed that clams from the 12-mm mesh bags were smaller (26.1 mm) than those from the 3-mm (33.1 mm) and 6-mm (32.2 mm) mesh bags. The results of the 12-mm mesh bags are moot, as only two animals survived (0.3%). ANOVA of survival data showed significantly greater survival occurred for clams stocked in the 3-mm mesh bags (62%) versus the 6-mm mesh bags (19%).

DISCUSSION

Surfclams demonstrated greater growth and survival when cultured in bottom cages as compared to the mesh-bag culture technique. Surfclams grown in cages achieved approximately twice the size of those grown in mesh bags at equivalent stocking densities (Table 1). The lowest size of clams in cages (46.5 mm in 600 clams/m^2 treatments) were 34% larger than the greatest size of clams in the mesh bag (34.6 mm in 500 clams/m^2) treatment. Atlantic surfclams had 1.5 times higher survival rate in cages than in mesh bags (Table 1).

Surfclams grow slower in mesh bags than in cages. The reason for this is unknown, but restricted water flow within the bags and increased exposure to turbulence are two possible explanations. Because the bags are lying on the sediment, water flow does not pass through the bags as it does for a cage. Instead water flows over the top of the bags. The cage sides cause a baffling effect thereby allowing food particles to fall out of the water column and consequently being more readily available to the clams. The physical pressure of the bag lying on the clam may have prevented efficient siphoning by the clam and hence, resulted in reduced feeding capabilities. In addition, currents appear to effect the bags more than the cages in this study. Although a few cages exhibited

TABLE 1.

Mean clam size in shell length and survival of Atlantic surfclams cultured at various stocking densities (clams per m^2) in bags and cages on an intertidal flat at House Creek, Little Tybee Island, Georgia. ANOVA and Tukey's SRT results are given for overall means where similar letters indicate means that are not significantly different.

	Cages			Bags		
Density Clams/m^2	Number Surviving	Survival (%) (p = 0.1994)	Mean ± SE Size in mm (p < 0.0001)	Number Surviving	Survival (%) (p = 0.0258)	Mean ± SE Size in mm (p < 0.0001)
100	76	76.0	54.8 ± 0.89	16	32.0	23.9 ± 0.96
100	57	57.0	51.5 ± 0.77	17	33.0	29.2 ± 0.87
100	53	53.0	52.2 ± 0.81	0	0	
overall	186	62.0a	52.8 ± 0.49a	33	22.0a	26.6 ± 0.80a
200	182	91.0	52.7 ± 0.63	0	0	
200	154	77.0	59.1 ± 0.59	56	56.0	30.5 ± 0.53
200	156	78.0	57.6 ± 0.78	55	55.0	35.2 ± 0.68
overall	492	82.0a	56.5 ± 0.45b	111	37.0ab	32.9 ± 0.49b
300	251	83.7	59.0 ± 0.58	85	56.7	27.1 ± 0.49
300	218	72.7	54.9 ± 0.73	94	62.7	31.8 ± 0.52
300	0	0		0	0	
overall	469	52.1a	57.0 ± 0.51b	179	39.8ab	29.4 ± 0.43c
400	356	89.0	49.7 ± 0.58	154	77.0	33.2 ± 0.63
400	305	76.3	53.9 ± 0.78	159	79.5	31.9 ± 0.57
400	339	84.8	43.9 ± 0.79	126	63.0	33.5 ± 0.63
overall	1000	83.3a	49.1 ± 0.53c	439	73.2ab	32.8 ± 0.41b
500	454	90.8	50.3 ± 0.87	210	84.0	32.1 ± 0.49
500	454	90.8	52.4 ± 0.78	169	67.6	35.1 ± 0.55
500	396	79.2	53.5 ± 0.79	172	68.8	36.7 ± 0.58
overall	1304	86.9a	51.0 ± 0.48c	551	73.5b	34.6 ± 0.35b
600	467	77.8	46.3 ± 0.60			
600	468	78.0	46.7 ± 0.64			
600	530	88.3	46.8 ± 0.54			
overall	1465	81.4a	46.5 ± 0.34d			

some wash-out of sediment, most mesh bag-lines were found twisted and in some cases completely relocated. Several times the lines had to be untangled and relaid. The physical movement of clams within bags by currents combined with the effects of increased densities resulting in a concentration of the clams into one end of the bag could retard growth. The effects of current on the bags may also explain why the higher bag stocking densities produced better clam growth and survival than that occurring in the lower density bags. The weight of the additional numbers of clams may provided a more stable environment within the bag.

In a similar study, northern quahogs, *Mercenaria mercenaria*, grew better in cages and in gravel filled trays than in the mesh-bag-line system (Walker 1997). Although growth was lower for northern quahogs cultured in mesh bags as compared to that for clams in cages or trays, survival of animals was found significantly higher in the mesh-bag treatment (Walker 1997). This was not the case for surfclams in this study. Atlantic surfclams in bags had 53% survival compared to 77% for those in cages. In most cases, greater growth and survival of surfclams occurred in bags with higher stocking densities. The increased biomass associated with higher densities may have provided a somewhat more stable environment consequently, the bags were not disturbed as much as less densely filled bags. During the northern quahog experiment at this same site (Walker 1997), bags were found filled with sediment at the end of that experiment; whereas, in this study, most bags were devoid of sediment.

The effects of stocking density on the growth of various bivalve species is well documented, whereby growth tends to be reduced

TABLE 2.

Mean clam size in shell length and survival of Atlantic surfclams cultured in 3-mm, 6-mm, and 12-mm mesh bags at the spring-low-water mark on an intertidal flat at House Creek, Little Tybee Island, Georgia.

Mesh Size (mm)	Number Surviving	Survival (%) (p = 0.0100)	Mean ± SE Size (mm) (p = 0.0623)
3 mm			
Bag 1	146	73.0	32.1 ± 0.51
Bag 2	125	62.5	32.6 ± 0.42
Bag 3	138	69.0	35.2 ± 0.54
Bag 4	85	42.5	32.5 ± 0.55
overall	494	61.8a	33.1 ± 0.27a
6 mm			
Bag 1	56	28.0	30.5 ± 0.53
Bag 2	55	27.5	35.2 ± 0.68
Bag 3	0	0	
overall	111	18.5b	32.2 ± 0.49a
12 mm			
Bag 1	2	1.0	26.1 ± 0.14
Bag 2	0	0	
Bag 3	0	0	
overall	2	0.3b	26.1 ± 0.14b

ANOVA (mean shell length; p = 0.0623 and survival; p = 0.0100) and Tukey's SRT results are given for overall means where similar letters indicate means that are not significantly different.

with increases in stocking density (Adams et al. 1994, Eldridge et al. 1979, Walker 1984, Goldberg 1989, Walker & Hurley 1995). Goldberg (1989) planted 15.7 mm Atlantic surfclams at 500, 1000, and 2000 clams/m^2 in bottom cages and after approximately four months, clams had grown to a mean size of 47.3 mm, 40.8 mm, and 32.0 mm L, respectively. In our study, 15.5 mm Atlantic surfclams planted in bottom cages at densities from 100 to 600 clams/m^2 showed significant decreases in growth between 200–300 and 400–500 clams/m^2 with slowest growth occurring at 600 clams/m^2 (Table 1). Interestingly, the lowest stocking density in bottom cages in our study (100 clams/m^2) yielded slower growth rates than the 200 and 300 clams/m^2 stocking densities, but had higher growth rates than did the 400 to 600 clams/m^2 stocking densities. Furthermore, the lowest stocking density of 100 clams/m^2 regardless of growout protocol yielded lower growth rates across both culture treatments compared to the 200 and 300 stocking densities (Table 1). The optimum stocking density for culturing Atlantic surfclams in Georgia to achieve maximum size is 300 clams/m^2; however, as discussed below, optimum stocking density for obtaining marketable size animals efficiently is 500 clams/m^2.

Aquaculture Recommendation

Aquaculture of Atlantic surfclams has excellent potential in coastal Georgia. Atlantic surfclams are easily spawned and reared under hatchery conditions. Seed can be grown in the hatchery to a field-planting size by mid-October, once ambient water temperatures decrease below 28°C. Ambient river temperatures in Georgia which may reach 31°C are lethal to the Atlantic surfclam, so seed must be reared in the hatchery at lower water temperatures throughout the summer months (Walker et al., 1997). Clams must be harvested by May to early June before ambient water temperatures reach 28°C. Atlantic surfclams are marketable within six months from planting. The results of this work show that cages can be seeded at different densities to produce an optimum sized clam (50 mm) for the lucrative steam or fried clam markets via a staggered crop method. Cages seeded at densities of 200 to 300 clams/m^2 can reach the targeted market size of 50 mm by March to April with cages seeded at 500 clams/m^2 achieving the same market size in May to June. Thus, by seeding at the optimum density for producing a maximum sized clam, the culturist can produce marketable clams early in the season. By seeding at 500 clams/m^2 for maximum production, clams will attain market size toward the end of the growing season (May to early June).

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COMPARISON OF RECRUITMENT FREQUENCY AND GROWTH OF SURFCLAMS, *SPISULA SOLIDISSIMA* (DILLWYN, 1817), IN DIFFERENT INNER-SHELF HABITATS OF NEW JERSEY

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ABSTRACT *Spisula solidissima* (Dillwyn, 1817) populations along the U.S. East Coast reflect episodic settlement and recruitment success. An analysis of growth bands in the shells of 1,005 surfclams collected from nine zones within 3 miles of the coast of New Jersey in 1993 revealed years of good recruitment, and allowed the construction of growth curves for each zone. The 1988 year class predominated in most areas, comprising 53.5% of all clams sampled, followed by the 1986 year class (8.3%), and the 1983 year class (5.0%). In the zones located farthest north, 3- to 5-year classes each represented at least 10% of the samples. All of the zones south of Barnegat Inlet were dominated by the 1988 year class, which accounted for 45% to 95% of the clams within these zones. The temporal patterns of recruitment across mile zones, depth gradients, bottom types, or catch densities did not appear to be significantly different. There was considerable variation among the growth curves for surfclams along the coast. An examination of the shell height at age 5 (H_5) for the 1988 year class in the northern and southern zones supports earlier findings that surfclams farther inshore grow more slowly and reach a smaller maximum size than clams farther offshore. The fact that the expected mean height at age one (H_1) for the 1988 year class was the same throughout all zones suggests a single, widespread settlement event along the coast in that year. An analysis of U.S. EPA surface and bottom temperature data (1979–1991) indicated that mean temperature for summer 1988 was lower than for all other years in the six northern zones, and was also relatively low in the three southern zones. Lower mean summer seawater temperatures are correlated with a higher frequency and persistence of coastal upwelling events, which in turn are associated with high larval abundances in the downwelling that immediately follows each upwelling. Thus, high recruitment may be the consequence of these hydrodynamic effects on larval supply, in addition to the effects of cool bottom temperatures in reducing predator impacts on recently settled surfclams.

KEY WORDS: surfclams, recruitment, growth, *Spisula solidissima*, New Jersey

INTRODUCTION

The surfclam, *Spisula solidissima* (Dillwyn, 1817), is one of the most abundant bivalves inhabiting eastern United States waters (Jones 1981a), and currently supports one of the largest shellfisheries on the east coast. It is found on the continental shelf in exploitable concentrations from the surf zone to depths of 50 m from Nova Scotia to North Carolina (Merrill & Ropes 1969, Abbott 1974, Wagner 1984, NEFSC 1998, NEFSC 2000), with lower densities offshore (Haskin 1978). In 1974, the highest concentration of surfclams in the New York Bight occurred off the southern coast of New Jersey in depths greater than 18 m (Garlo 1982), but in recent years the highest concentrations have occurred off northern New Jersey (NEFSC 1998, NEFSC 2000).

In 1977, approximately 80% of all U.S. clam products were from surfclams (Loesch & Ropes 1977, Wagner 1984). Surfclams have been heavily fished since the 1940s, and overfishing led to a decline in surfclam densities (Jones 1981a). Although *S. solidissima* can settle in very high numbers (e.g., 250,000 per m^2 at an inshore site in 1993, Weissberger 1998), most populations have nearly complete mortality by late summer and fall in many years (Haskin et al. 1979, Garlo 1982, Weissberger 1998). These mortalities have been attributed to hypoxia and anoxia, which destroyed up to 62% of the New Jersey surfclam resource in 1976 (Ropes et al. 1979, Garlo 1982), and to predation (Garlo 1982, Botton & Haskin 1984, Mackenzie et al. 1985, Weissberger 1998, Weissberger 1999). Weinberg (1999) has shown that although U.S. Exclusive Economic Zone (EEZ) populations off Delmarva and New Jersey had only 2- or 3-year classes in 1978 following the widespread hypoxia in 1976, additional year classes recruited in relatively low numbers throughout the next two decades.

The variability in successful recruitment of this species in inshore areas is due in large part to post-settlement predation (MacKenzie et al. 1985, Weissberger 1998). Previous research has shown differences in growth rates between inshore and offshore clams, with inshore clams growing more slowly, reaching a smaller maximum size, and having shorter life spans than offshore clams (Jones et al. 1978, Jones 1980, Ambrose et al. 1980). These growth differences have been attributed to density differences and differences in annual mean temperatures (Ambrose et al. 1980, Jones 1981b, Weinberg 1999). In the present study we examined only populations that had been previously described as inshore populations (Giust 1993). This research was part of an ongoing effort to determine where and when settlement and good growth occur off New Jersey. We are testing the hypothesis that there have been successful year classes of surfclams, other than the post-anoxia year classes, that have recruited off the coast of New Jersey to support the fishery.

MATERIALS AND METHODS

Stations Sampled

Surfclams were collected from 26 stations along the coast of New Jersey (Fig. 1). Two stations were located at the Rutgers University's Long Term Ecosystem Observatory site in 15 m depth (LEO-15) and the other 24 were a subset of 318 stations in a survey by the New Jersey Department of Environmental Protection (NJ DEP) in July and August 1993. The stations chosen by the NJ DEP were assigned using a stratified design (Giust 1993) based on previous annual surveys and were not randomly distributed in the zones, located 1, 2, and 3 miles off the NJ coast (Fig. 1). The 24 stations were chosen based on their geographic location and the probability of gaining a sufficient number of surfclams. Whenever possible, our stations were selected along a latitudinal line perpendicular to the shore.

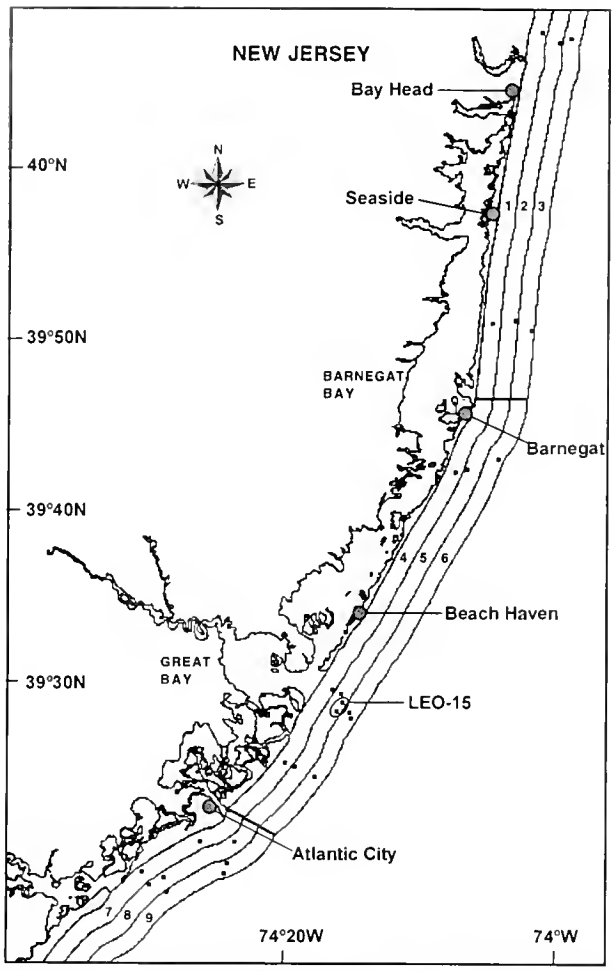

Figure 1. Coast of New Jersey indicating the stations where surfclams were sampled in 1993. Lines paralleling the coast delineate the 1-mile zones for NJ DEP sampling. Numbers indicate the zone delineations. Cities and towns indicate the origins of U.S. EPA weekly transects measuring surface and bottom temperature and oxygen concentration in May to September, 1979–1991.

Clams were collected by a commercial hydraulic clam dredge equipped with a 72-inch knife. The dredge floor was lined with Springfield fencing with 51-mm square mesh. Ring diameter in the metal bag at the cod end of the dredge was 72 mm (Giust 1993). The smallest clam that was caught with this dredge and measured was 33 mm in length. We followed the same assumptions as Weinberg (1999) in that clams smaller than this were completely missed during sampling and that other clams that could pass the ring size of the dredge were not sampled quantitatively. One tow was taken per station, and was assumed to randomly sample surfclams fully retained by the dredge in that area. Clams were haphazardly selected from a one-bushel subsample at each station (sample size ranged from 6–69). These surfclams were shucked on board and the shells were returned to the laboratory for age and growth determination. The data from the different stations were analyzed by zones as described below.

Recruitment and Growth Curve Determinations

The shells were cleaned and height was measured. Because the clams were large, they were not embedded prior to cutting and counting the growth lines (Jones, pers. comm.). For consistency, whenever possible, only the right valve was cut for age and growth determinations. On some occasions it was necessary to use the left valve when the right valve broke during sectioning. A single cut was made with a diamond saw from the umbo to the ventral margin along the axis of maximum height (Jones et al. 1978). The shell edge was polished on a lapidary wheel for 2 to 3 min until the edge was smooth. The dark internal growth lines were counted and used to back-calculate the year of settlement for each of the clams since Jones et al. (1978) have shown that the growth lines are annual.

The height at each age was measured directly from the dark, internal growth lines, which were traced from the chondrophore to the outer shell surface. The shell was examined under a dissecting microscope at 60× or 120× (objective and eyepiece magnification) and the exit point of the growth line was marked with a pencil. The straight-line distance from the umbo to the point of exit of each growth line at the shell's outer surface (Jones et al. 1978) was measured to the nearest 0.1 mm with calipers. In this way, a series of height-at-age measurements were obtained from each individual.

Analysis of Data

Data were analyzed as in Cerrato and Keith (1992). A von Bertalanffy curve was fit to the shell height vs. age data by nonlinear least-squares analysis using SAS for all clams measured in each of the zones (Cerrato 1990). The algebraic form of the von Bertalanffy curve used in these analyses was

$$h = H_1 + (H_{max} - H_1)(1 - x^{t-1}), \quad (1)$$

where h is the shell height at age t, H_1 is the expected mean height at age 1, H_{max} is the asymptotic maximum size, and x is the expected fractional reduction in annual increment growth (Cerrato & Keith 1992). Parameter estimates were determined for H_1, H_{max}, and x. The growth parameter, k, which is a measure of how fast a population reaches its maximum size, was calculated as follows:

$$x = e^{-k}. \quad (2)$$

The 1988 year class was the predominant year class in most of the samples. Therefore, we ran a separate analysis on the representatives of that year class. Because individuals in this year class had not yet approached their asymptotic growth stage, the algebraic form of the von Bertalanffy equation used for this analysis was (Cerrato & Keith 1992)

$$h = H_1 + (H_5 - H_1)(1 - x^{t-1})/(1 - x^4), \quad (3)$$

where H_5 is the height at age 5 and the other parameters are the same as in equation (1). Parameter estimates for H_1, H_5, and x were determined and analyzed.

Analyses of the parameter estimates from the nonlinear regression were done with likelihood ratio tests, and 95% confidence intervals were generated by projecting the likelihood regions onto the appropriate axes (Cerrato 1990, Cerrato & Keith 1992). Data were analyzed by zone, with each zone containing two to five stations. The experiment-wise error rate was 0.05, and the per comparison rate was 0.05/p, with p = 36, the number of comparisons.

Temperature and Dissolved Oxygen Data

Temperature and dissolved oxygen data, taken from 1 and 3 miles off the New Jersey coast for years 1979 to 1991, were

obtained from the U.S. EPA (1994). Surface (1 m below surface) and bottom (1 m above bottom) data were collected as part of the U.S. EPA helicopter water quality sample collection for the New York Bight area. Sampling occurred weekly during the summer each year from approximately mid-May to late September. The weekly values were averaged to get a mean surface and bottom temperature for each station and station values within a zone were averaged. The sampling entailed nine transects extending east from the NJ coast, with five stations in each transect. The only transects used were those from Bayhead (corresponding to zones 1 and 3), Seaside (zones 1 and 3), Barnegat (zones 4 and 6), Beach Haven (zones 4 and 6), Atlantic City (between zones 4 and 7, and 6 and 9), and Strathmere (zones 7 and 9) (Fig. 1; Chintala 1997).

RESULTS

Recruitment History of S. solidissima

The 1988 year class was predominant in the samples (53.5% of all clams sampled), followed by the 1986 year class (8.3%), and the 1983 year class (5.0%). Zones 1, 2, and 3 had surfclams from many different year classes (Fig. 2, A–C). Zone 1, the zone located furthest north and within 1 mile of the shore, was the only zone where the 1983 year class was the most abundant (Fig. 2A). The 1986, 1987, and 1989 year classes were also well represented (>10%) in this sample. Zone 2, between miles 1 and 2, was dominated by five year classes, 1982, 1983, 1986, 1987, and 1988 (Fig. 2B). The northern zone farthest offshore, zone 3, was more clearly dominated by the 1986 year class (Fig. 2C), but several other year classes were represented in the sample.

Most of the other zones (4–8), were dominated by the 1988 year class (Fig. 2, D–H). This year class accounted for 45% to 95% of the clams within these zones. Although the 1988 year class was the most prevalent in zone 9 (33%), there were also a large number of 1976 and 1977 year class clams in these samples (29.6% and 18.5%, respectively; Fig. 2I). Recruitment across mile zones was similar since the same age-class pattern was seen in zones 1 through 3, zones 4 through 6, and zones 7 through 9 within each mile; however, towards the south and farther offshore, the number of clams in the post-anoxia year classes (1976–1978) became more prominent (Fig. 2I). The northern stations appeared to be represented by a wider range of year classes than farther south (Fig. 2). South of Barnegat Bay, the samples appeared to be overwhelmingly dominated by the 1988 year class.

Growth Curves of S. solidissima

The likelihood ratio tests indicated that the von Bertalanffy growth curves were the same for zones 1 and 4 and for zones 8 and 9. For all the other pairwise zone combinations, the growth curves were significantly different from one another (Table 1). The growth in the first 3 to 4 y of life was similar for the nine zones (Fig. 3). The growth curves up to age 5 for zones 5 through 8 were lower and more variable than those from other zones, probably due to the dominance of the 1988 year class (Fig. 3).

The expected mean height at age 1 (H_1) was the same for zones 1 and 4 through 9 (Fig. 4A). Clams in zones 2 and 3 were significantly smaller at age 1 than those in the other zones. The expected maximum height (H_{max}) varied among zones and had smaller confidence intervals than H_1 (Fig. 4B). Again, H_{max} in zones 2 and 3 were not significantly different from one another, but were greater

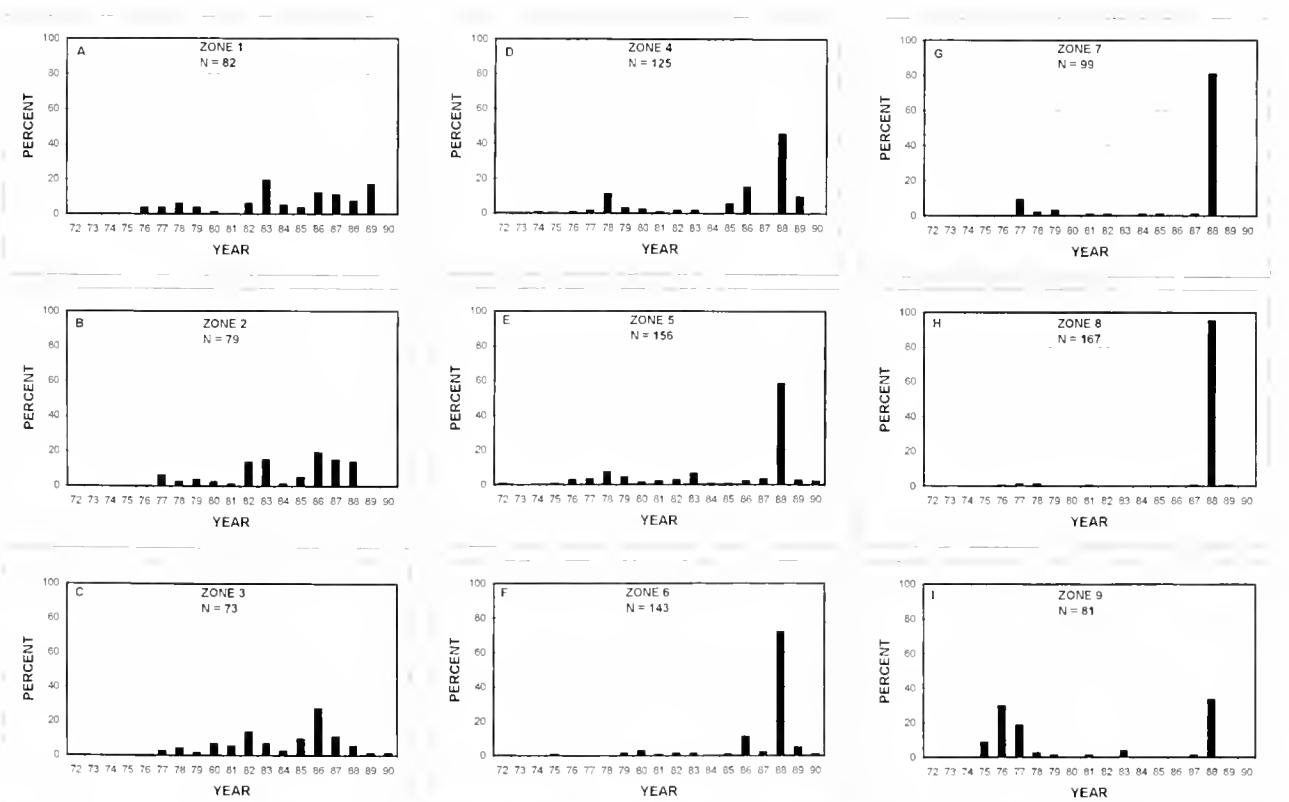

Figure 2. A-I. Percentage of surfclam sample in each year class for nine inshore zones off New Jersey. N indicates sample size for that zone.

TABLE 1.

Likelihood ratio tests comparing the von Bertalanffy growth parameters among zones (1–9) off the New Jersey coast. The test statistic is $-2\log(\Lambda)$ where Λ is the likelihood ratio. Significant outcomes are underlined. The maximum likelihood estimates under the null hypotheses were calculated by iteratively reweighted least squares due to heteroscedasticity in the error variances between stations. The experiment-wise error rate for each hypothesis was 0.05, and the per comparison error rate was $\alpha = 0.05/p$, where p is the total number of pairwise comparisons (based on the Bonferroni inequality). A paired comparison was significant if $-2\log(\Lambda) > n\log[1 + (q/\nu)F^{\alpha}_{q,\nu}]$, where q is the number of parameters being compared, n is the total sample size, $\nu = n - 6$, and $F^{\alpha}_{q,\nu}$ is the upper α percentile point of the F distribution with q and ν degrees of freedom. (Cerrato 1990, Cerrato & Keith 1992).

Stn	1	2	3	4	5	6	7	8	9
H_0: H_1, H_{max}, x equal, all year classes									
1	–								
2	311.18	–							
3	182.11	19.56	–						
4	4.40	341.16	206.71	–					
5	54.96	387.29	246.74	45.10	–				
6	289.70	130.98	130.60	303.88	271.39	–			
7	229.49	581.80	425.90	187.93	92.68	396.81	–		
8	75.63	170.78	104.51	110.27	273.30	322.66	503.23	–	
9	93.66	209.03	118.58	132.22	215.06	323.21	463.11	6.59	–
H_0: H_1 equal, all year classes									
1	–								
2	14.15	–							
3	17.99	0.53	–						
4	0.296	12.18	16.00	–					
5	2.49	23.84	27.43	5.08	–				
6	2.03	21.35	25.13	3.94	0.003	–			
7	5.64	29.12	32.41	9.63	0.72	0.45	–		
8	0.92	21.04	24.69	2.79	0.70	0.56	3.19	–	
9	4.79	25.35	29.10	7.33	1.03	0.77	0.17	2.78	–
H_0: H_{max} equal, all year classes									
1	–								
2	172.95	–							
3	92.43	8.79	–						
4	1.48	198.38	110.83	–					
5	7.83	117.76	53.45	15.69	–				
6	283.62	71.40	107.43	301.10	241.02	–			
7	2.73	158.40	92.88	0.44	15.04	282.23	–		
8	0.15	113.00	58.75	1.68	3.06	240.74	2.83	–	
9	11.57	142.84	64.38	22.70	0.008	255.36	19.03	3.94	–
H_0: k equal, all year classes									
1	–								
2	8.88	–							
3	2.47	1.27	–						
4	0.08	10.71		–					
5	31.36	2.26	7.51		–				
6	212.71	111.17	124.86	225.73	122.19	–			
7	37.62	5.04	11.50	43.20	1.08	95.96	–		
8	8.52	28.11	15.05	7.41	67.90	264.29	73.27	–	
9	1.55	15.44	6.41	1.00	43.64	228.69	49.82	2.71	–
H_0: H_1, H_5, x equal, 1988 year class									
1									
2	31.36	–							
3	21.08	2.88	–						
4	0.17	49.91	21.97	–					
5	13.36	80.04	29.89	83.42	–				
6	24.81	97.80	34.14	161.01	35.18	–			
7	16.13	83.08	30.84	104.23	8.98	29.12	–		
8	30.23	33.53	18.49	235.18	446.30	599.90	465.58	–	
9	39.51	7.89	8.73	124.96	175.68	212.12	179.30	39.82	–

continued on next page

TABLE 1.

continued

Stn	1	2	3	4	5	6	7	8	9
H_0: H_1 equal, 1988 year class									
1	–								
2	0.118	–							
3	0.075	0.003	–						
4	0.003	0.103	0.048	–					
5	1.480	2.448	0.786	9.312	–				
6	0.303	0.906	0.319	2.275	2.998	–			
7	0.232	0.704	0.329	1.304	5.851	0.416	–		
8	0.151	0.557	0.264	0.922	8.621	0.896	0.300	–	
9	0.004	0.119	0.066	–0.006	3.823	0.880	0.523	0.339	–
H_0: H_5 equal, 1988 year class									
1	–								
2	24.63	–							
3	19.73	2.77	–						
4	0.022	39.61	20.48	–					
5	11.69	75.78	29.30	68.15	–				
6	24.45	95.04	33.79	155.86	30.66	–			
7	15.09	81.25	30.62	93.87	2.40	18.79	–		
8	3.61	26.67	16.52	17.10	225.41	389.72	289.83	–	
9	21.68	1.48	5.92	62.37	147.70	192.40	161.16	39.215	

than all but zone 6. Zones 1, 4, 7, and 8 were not significantly different. Likewise, zones 5, 8, and 9 were not significantly different from each other, and zones 1 and 5 were the same (Table 1). The largest H_{max} was in zone 6, one of the offshore zones. The clams with the smallest H_{max} were found in zones 1, 4, and 7 within 1 mile of the New Jersey coast (Fig. 4B).

The growth parameter k ranged from 0.164 to 0.396 (Fig. 5). Zone 6, which had the highest H_{max}, also had the lowest k, while zone 8 had the highest k. The statistical analysis showed that the k value for zones 1, 3, 4, and 9 were not significantly different from one another, nor were zones 2, 3, and 5 different from each other.

Growth Curves of 1988 Year Class of S. solidissima

Testing if all the von Bertalanffy parameters were equal for all zones showed the following homogeneous groups: zones 1 and 4; zones 2, 3, and 9; zones 1 and 5; and zones 5 and 7 (Fig. 6; Table 1). The analysis of H_1 showed that there were no significant differences among the zones (Fig. 7A; Table 1). The largest clams at age one were in zones 5 and 6. The greatest variability in H_1 was in zones 1 through 3, zones that also had the lowest number of 1988 year class clams (Fig. 7A). The estimate of the height at age 5 showed that clams from zones 2, 3, and 9 were similar and were also the largest (Fig. 7B). The smallest clams were in zones 5, 6, and 7, with only zones 5 and 7 not significantly different from each other (Table 1). Zones 1 and 4 were the same as were zones 1 and 8 (Fig. 7B).

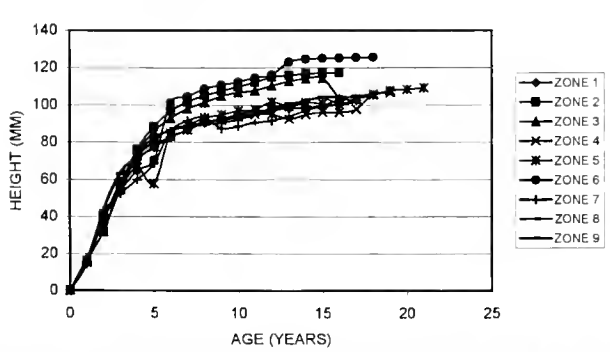

Figure 3. Mean height (millimeters) at each age for all clams collected at each of the stations in the nine zones off the coast of New Jersey.

Temperature and Dissolved Oxygen Data

Only the summer means (from mid-May to late September) for the surface and bottom temperatures and dissolved oxygen measurements for the stations that corresponded to clam sampling stations were examined. The data for stations in each of the inshore zones (1, 4, and 7) and the offshore zones (3, 6, and 9) were averaged. In zones 1 and 3, mean summer surface and bottom temperatures tracked each other closely from 1979 to 1991, with surface temperatures being 4°C to 5°C above bottom temperatures in every year (Chintala 1997). In zones 1, 3, 4, and 6 there were lower average temperatures in 1988 than in all other years (Fig. 8). At the southern border of zones 4 and 6, and in zones 7 and 9, temperatures were low in 1988, but not uniquely so, during this 13-year period (Fig. 8). Dissolved oxygen data showed very little variation at any of the sites, varying by less than 3 mg/L throughout the 13 y, and there was no evidence of widespread, long-lasting hypoxia (see Chintala 1997 for complete temperature and dissolved oxygen data).

DISCUSSION

S. solidissima commonly experiences episodic recruitment (Loesch & Ropes 1977) and age-class dominance in dense populations (Murawski & Serchuk 1989). Since the anoxic event off the New Jersey coast in 1976 that killed 62% of the New Jersey surfclam resource in one area (Garlo 1982), it had been thought that the fishery was supported predominantly by the 1976 and

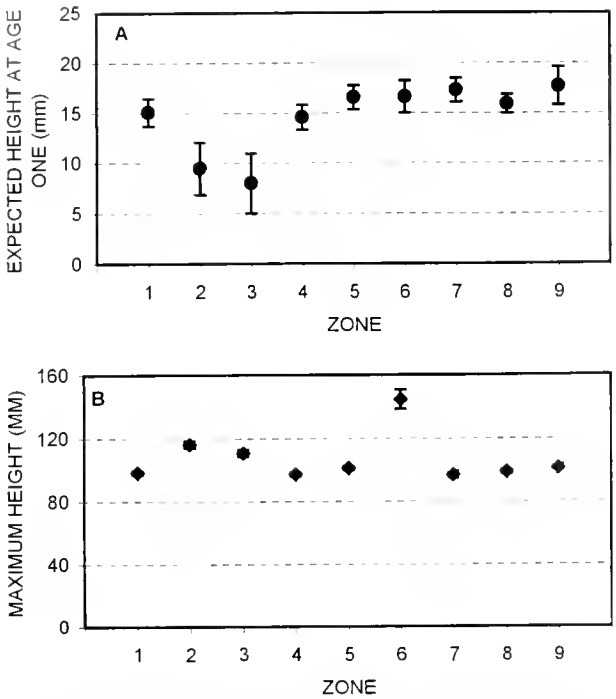

Figure 4. A. Expected height at age 1 for all clams sampled in each of the nine zones as determined by von Bertalanffy analysis. Values were determined by nonlinear regression. Bars indicate 95% confidence intervals for each estimate. B. Expected maximum height for all clams sampled in each of the nine zones determined by von Bertalanffy analysis. Values were determined by nonlinear regression. Bars indicate 95% confidence intervals for each estimate.

1977 year classes (Murawski & Serchuk 1989). These year classes survived, most likely because of the anoxia-related mortality of the main predators of juvenile *S. solidissima*, including the crab, *Ovalipes ocellatus*, and the starfish, *Asterias forbesi* (Garlo 1982, MacKenzie et al. 1985). Cerrato and Keith (1992) found that in 1988, the surfclam populations in estuarine waters off Long Island Sound, which were not affected by the hypoxia further south, were instead dominated by the 1983 and 1984 year classes.

Previously, it had been thought that there was little or no recruitment to the fishery inshore or offshore subsequent to the 1976

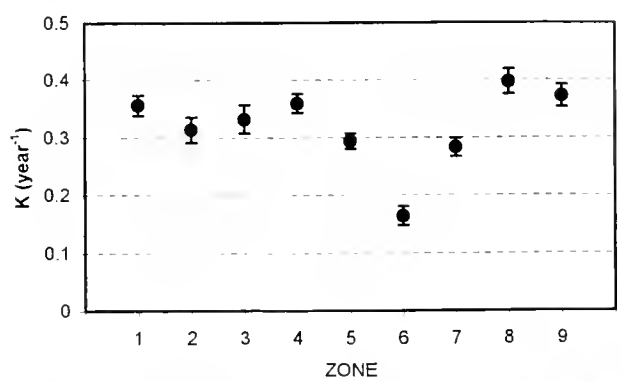

Figure 5. The time each clam population takes to reach maximum size (k) for all clams collected in each zone. Values were determined by Eq. 2 in the text. Bars indicate the 95% confidence intervals for each estimate.

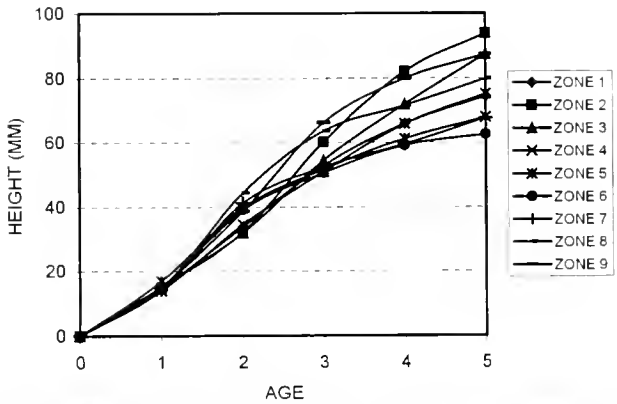

Figure 6. Mean height (millimeters) at each age for the 1988 year class collected from each of the nine zones off the coast of New Jersey.

and 1977 cohorts (NEFSC 1989, Murawski & Serchuk 1989). In the area designated as Northern New Jersey (area from the New York/New Jersey border south to 39°10′ or 30°30′ latitude, depending on distance offshore (Serchuk et al. 1979)), NMFS determined in a 1992 EEZ assessment study that there was no evidence of any substantial recruitment since 1976-1977. However, in southern New Jersey, they determined that there was some recruitment into the fishery between the years 1986 and 1992 (Weinberg

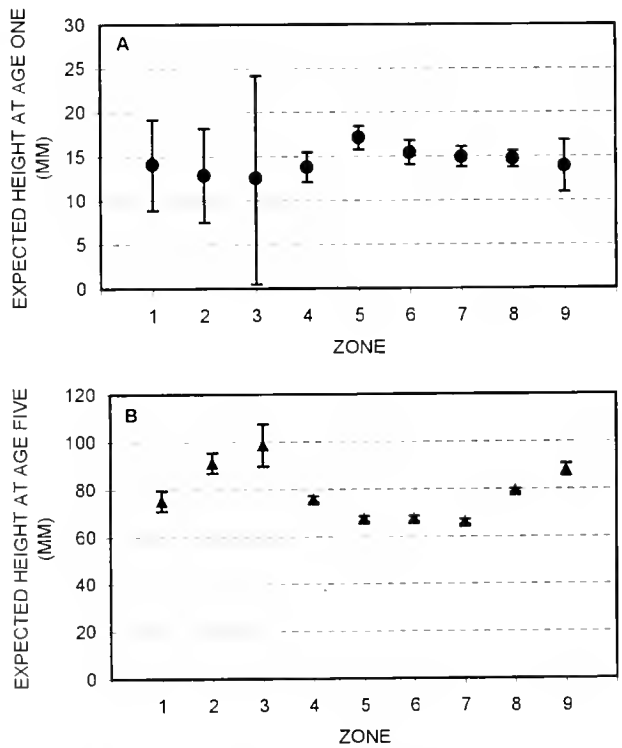

Figure 7. A. Expected height at age 1 calculated by von Bertalanffy analysis for the 1988 year class clams sampled in each of the nine zones. Values were determined by nonlinear regression. Bars indicate 95% confidence intervals for each estimate. B. Expected height at age 5 calculated by von Bertalanffy analysis for the 1988 year class clams sampled in each of the nine zones. Values were determined by nonlinear regression. Bars indicate 95% confidence intervals for each estimate.

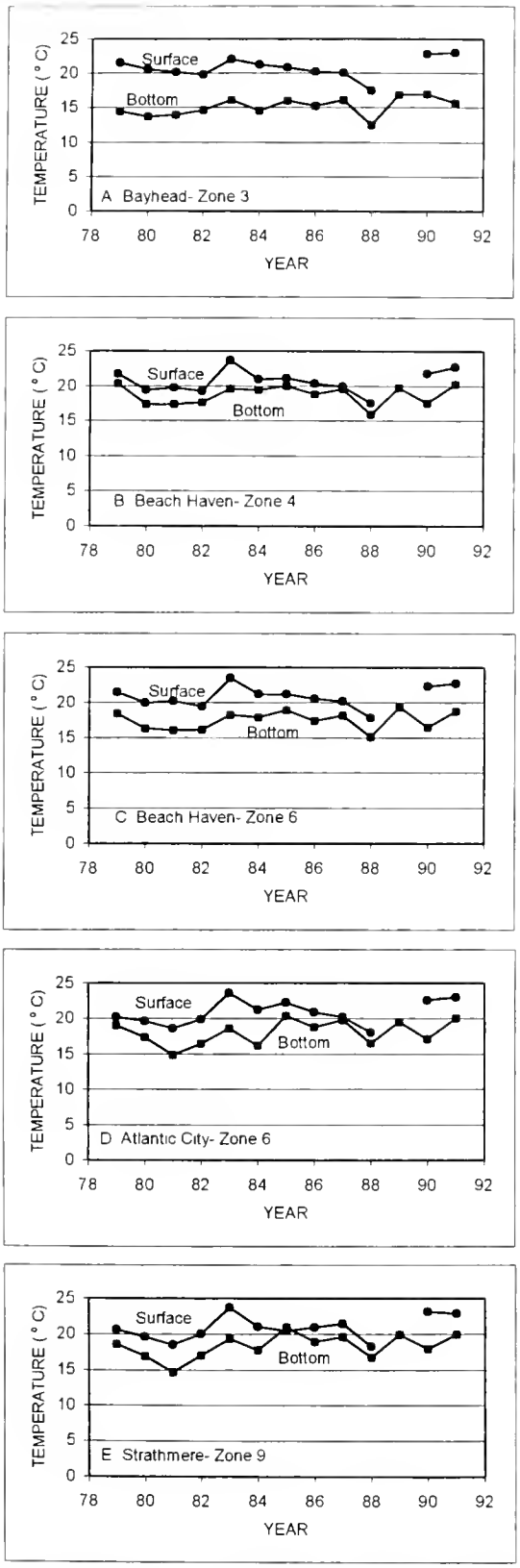

Figure 8. Average summer temperature for surface (1 m below) and bottom (1 m above bottom) waters off the coast of New Jersey from 1979 to 1991 for selected transect locations. Data courtesy of the U.S. EPA (1994).

1993). The 1988 year class, which dominated the central and southern regions of our sampling regime, falls within this time frame. These results suggest that a significant settlement event occurred off southern New Jersey in both the inshore and EEZ populations during this time. Recent data have shown that the EEZ surfclam fishery has indeed been supported by multiple year classes throughout the 1980s and 1990s (Weinberg 1999). In an area offshore from our sampling stations in NJ, it appeared that after the 1976 hypoxia event, there were several years when surfclam recruitment was relatively high, followed by several years of relatively low recruitment. A 1997 assessment showed that the 1976 and 1977 cohorts were then only a small component of the population, which was represented by at least 19 year classes (Weinberg 1999).

The results from our inshore collections suggest that a new year class (1988) had recruited to the fishery, at least off the central and southern New Jersey coasts. Six of the nine zones sampled were dominated by this year class, and in some zones, such as 5, 7, and 8, it was the only major year class present. The only substantial remnant of the post-anoxia population (1976 and 1977 year classes) was seen in zone 9.

The NJ DEP has reported that the inshore surfclam population structure in 1992 was still dominated by the 1976 and pre-1976 year classes that survived the anoxic conditions (Giust 1993), the average shell length of the surfclams being 94.1 mm. To compare our results with those from the NJ DEP reports, we converted the surfclam shell heights to lengths using a height:length ratio for each station. The mean length of the dominant 1988 year class in our samples at age 4 (corresponding to 1992) varied between 60 and 80 mm, depending on the station, but it was difficult to compare these values with the overall mean in the NJ DEP study since their clams were not aged.

The issue of when the dark growth bands used in age determinations are laid down in the shell has been extensively discussed by Jones et al. (1983), who used oxygen and carbon stable isotopic analyses in a single surfclam shell taken from 9 to 10 m depth off the Barnegat Light (at the southern border of zone 1 in the present study) to examine the correlation between shell increments and monthly average sea surface temperatures. He concluded that in this inshore environment, shell growth is most rapid in spring and early summer, slows in late summer and fall, and is extremely slow or nonexistent in the winter. This agrees well with our own observations at the LEO-15 site on Beach Haven Ridge (zone 5 in this study; Weissberger 1998, Ma 1999, Grassle unpubl. data). Past research has suggested that the age bands were laid down annually in surfclams (Jones et al. 1978, Jones et al. 1983). One notable feature of Jones (1983) study was the large shell length (~56 mm) at age 1 of the individual surfclam that was studied. Jones (1983) noted that the values of $\delta\ ^{18}O$ predicted from the monthly sea surface temperatures and the measured oxygen isotope values in the first growth increment agreed well except during the months of January through March, and that it indeed represented only 1 year's growth. If settlement of this individual had occurred in the early summer, as is likely off Barnegat Light, and the first clearly visible growth line was laid down in the winter of the following year, the first growth increment may, in fact, have represented growth over 16 to 18 mo, making the length at "age 1" less anomalous. In the present study, the expected mean shell heights at age 1 (H_1) were considerably lower (ranging from 8.0 mm in zone 3 to 17.5 mm in zone 9, corresponding to shell lengths

of ~10.3 and 22.0 mm, respectively), than the previous the shell height at age one (56 mm) of the individual in Jones (1983) study. The first band is often incomplete and faint in surfclams, and these lines may have consistently been missed or ignored in earlier studies, resulting in apparently large shell heights at age one (Jones 1983, Ropes 1985). Some error could have been introduced in the determination of the first age band, but the most likely factor to affect H_1 would be the time of settlement of the larvae. Larvae that settle later in the year would be expected to have a smaller H_1. One of the most striking characteristics of the 1988 year class is the fact that H_1 was the same in all the zones. This suggests a single, widespread settlement event within 3 miles of the coast in 1988.

It has also been suggested previously that growth lines might be laid down at the time of spawning (Jones 1980). There does not seem to be any positive evidence for this in surfclams living off New Jersey, and contrary to previous suggestions that surfclams first reach sexual maturity at age 2 (Ropes 1979), inshore surfclams as young as 3 mo of age and 5 mm in shell length were found to be sexually mature (Chintala & Grassle 1995). It is likely that inshore and offshore surfclams spawn at different times each year, with the inshore clams spawning as soon as bottom temperatures increase above 12°C to 13°C in the late spring or early summer. The offshore clams probably do not spawn until bottom temperatures reach this level as a consequence of the breakdown of the thermocline in the early fall, or because of storm mixing (Haskin et al. 1979, Weissberger 1998). Benthic community studies at LEO-15 indicate that surfclam settlement occurs not only in the early summer, but at low levels in the fall (e.g., October and December of 1993; Weissberger 1998), and molecular identifications of bivalve larvae indicate the presence of surfclam larvae in the water column in winter (December and February of 1995/1996; Gregg, Tucker & Grassle, unpubl. data). Thus, it seems likely that the larvae produced by spring/early summer spawning of inshore surfclams settle in early summer, while those produced in the fall may settle in the winter and grow very little prior to the following summer. As a result, the first winter growth line may therefore only be visible in the shells of clams that settle in the summer.

A number of hypotheses to explain slower growth (and therefore a decrease in von Bertalanffy parameters) from New Jersey and Delmarva surfclam populations in the EEZ were put forward by Weinberg and Helser (1996) and Weinberg (1999). Among these were the rapid population increase following the 1976 anoxia event, which might have increased intraspecific competition; commercial size-selective harvesting; and the use of hydraulic dredges that damage clams, expose them to predators, resuspend bottom material, and potentially decrease food quality. Although these hypotheses were for surfclam growth differences from offshore regions that do not overlap with our sampling area, each of these hypotheses could also explain the differences in growth curves in the inshore New Jersey regions that we sampled, since the 1976 anoxic event also affected many inshore regions of New Jersey, and hydraulic clam dredges are used to harvest clams within the 3-mile New Jersey coastal zone.

Results suggest uniformly slower growth along the northern coast of New Jersey within 1 mile of shore, and off southern New Jersey 2 to 3 miles offshore. This slower growth could be a lingering effect of competition from the 1976 and 1977 year classes, even though those year classes are now essentially gone from the populations except in zone 9. The explanation for this growth differential is complex since there is a spatial and temporal component to the variability. In addition, the smallest maximum heights were from surfclams within 1 mile of shore (zones 1, 4, and 7), while the largest maximum heights were in populations 2 to 3 miles offshore (zones 2, 3, and 6). Also, the lowest k (how fast the population reaches maximum size) was in zone 6, suggesting a less rapid reduction in annual growth with age, while the highest k was in zones 1, 4, 8, and 9, suggesting a rapid reduction in annual growth with age. These findings do not support previous results that inshore clams always grow more slowly and reach a smaller maximum size than those farther offshore (Jones et al. 1978, Ambrose et al. 1980, Jones 1980, Wagner 1984, Cerrato & Keith 1992).

There were also many differences in the expected mean height at age 5 (H_5) among the zones, suggesting that environmental or growth conditions are different among zones. The fastest growth for the 1988 year class was in zones 2, 3, and 9, while the slowest growth appeared to be in zones 5, 6, and 7. The H_5 for this 1988 year class in zones 1 and 7 were less than in zones 3 and 9, suggesting that in the northern and southern zones the surfclams furthest offshore had grown faster and reached a larger size than those inshore. This pattern was not supported in the central zones (zones 4–6). These H_5 differences could be related to density, since Cerrato and Keith (1992) found that H_5 decreased significantly with surfclam density in the waters off Long Island. Density had no effect on the H_1 (0–300 clams per m^2), but a significant decrease in H_5 was found with densities >100 per m^2. In the present study, some of the largest clams were found in zones 2 and 3, and likewise, the highest H_5 for the 1988 year class clams was in these zones. In a study off the Delmarva Peninsula in an area closed to commercial harvesting from 1980 to 1991, Weinberg (1998) found that the intensity of apparent competition among surfclams was greatest in areas of the highest density. This competition was evident in a strong negative relationship between mean surfclam length and density of clams aged 5 to 12 y. Unfortunately, in the inshore New Jersey areas where we sampled, intense harvesting could have obscured the relationship between density and growth rate by disturbance from the hydraulic clam dredges and removal of large numbers of clams from an area in a short period of time (Weinberg 1998).

Reasons for the success of the 1988 year class are likely to be multiple and complex. The stations were located within 3 miles of the New Jersey coast. There did not appear to be a pattern of settlement within the mile zones. There did appear to be a latitudinal gradient, however, with the northern regions being dominated by numerous year classes and the more southern zones being dominated overwhelmingly by the 1988 year class. Gradients in age-structure have been identified for other surfclam populations along the east coast. Cerrato and Keith (1992) reported that east of Fire Island Inlet, New York, on the south shore of Long Island, the surfclam population was characterized by a wide range of ages with no evidence of age class dominance, but in Long Island Sound and at Long Beach, one or two age classes dominated and there were few or no clams >8 y old.

Temperature records from the U.S. EPA (1994) indicate that the mean summer surface temperatures in 1988 were the lowest in 13 y over much of the study area (ranging from 17°C to 18°C), and mean bottom temperatures were 12°C to 15°C in zones 1 through 6. These low mean summer bottom and surface temperatures likely reflect the frequency and persistence of near-coastal

summer upwelling events. The differences among the zones alongshore in different years are not surprising considering the way in which the upwelling nodes along the New Jersey coast are distributed (Glenn et al. 1996). Downwelling that follows the upwelling events in the vicinity of the LEO-15 site in zone 5 have been shown to be associated with relatively brief pulses of very high concentrations of *S. solidissima* larvae (Ma 1997), and subsequent larval settlement (Ma unpubl. data). Thus, it is not surprising to find a correlation between a measure of upwelling (low mean summer surface or bottom temperature) and surfclam year class strength. Good growth years and good recruitment years for *S. solidissima* have previously been associated with cool rather than warm years (Jones 1980). Generally, cool summers and frequent inner-shelf upwelling events may be associated not only with high larval settlement, but also with reduced mortality due to changes in predator abundance or predator activity (Weissberger 1998).

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CHROMOSOMAL MAPPING OF THE VERTEBRATE TELOMERIC SEQUENCE (TTAGGG)N IN FOUR BIVALVE MOLLUSCS BY FLUORESCENCE IN SITU HYBRIDIZATION

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ABSTRACT The presence and chromosomal location of the vertebrate telomeric sequence (TTAGGG)n was studied in four bivalve molluscs: the eastern oyster, *Crassostrea virginica* Gmelin; the mangrove oyster, *Crassostrea rhizophorae* Guilding; the hard clam, *Mercenaria mercenaria* Linnaeus; and the dwarf surfclam, *Mulinia lateralis* Say, using fluorescence *in situ* hybridization (FISH). Metaphase chromosomes were prepared from embryos, and the vertebrate telomeric probe was labeled with digoxigenin and detected with fluorescein labeled anti-digoxigenin antibodies. Positive FISH signals were detected in all four species. Signals were restricted to telomeric regions of all chromosomes, and no interstitial sites were observed. Results of this and previous studies suggest that the vertebrate sequence is likely the telomeric sequence for all bivalve molluscs, which so far are the only invertebrates showing the vertebrate lineage in telomeric DNA.

KEY WORDS: FISH, chromosome, telomeric sequence, mapping, evolution, mollusca

INTRODUCTION

Telomeres, the specialized protein-DNA structures at ends of linear chromosomes, are essential for the stability of all eukaryotic chromosomes. Telomeres protect chromosomes from DNA degradation and end-to-end fusion, and may play important roles in aging and oncogenesis (Blackburn 1991, Harley et al. 1990, Hastie et al. 1990). In most organisms, telomeric DNA consists of tandem repeats of very simple sequences (6–10 bp), which are often conserved across taxa. All vertebrates studied so far have the same telomeric sequence, (TTAGGG)n (Meyne et al. 1989, Zakian 1995). The vertebrate sequence is shared by some other organisms such as *Trypanosoma* protozoa, several slime molds and fungi (Zakian 1995). In invertebrates, telomeric sequences are not extensively studied, but available data show more diversity. Most insects studied have a telomeric sequence of (TTAGG)n, while *Drosophila* flies and some other insects do not have either the insect or the vertebrate telomeric sequence (Okazaki et al., 1993). Telomeric sequence in *Drosophila* flies consists of transposable elements, not simple repeats. Nematodes have a telomeric sequence of (TTAGGC)n (Zakian 1995). Two groups of ciliates, *Tetrahymena* and *Oxytricha*, have different telomeric sequences of (TTGGGG)n and (TTTTGGGG)n, respectively.

Telomeric sequences in molluscs are largely unknown. Only four bivalves have been studied so far, and interestingly all four bivalves contain the vertebrate sequence, (TTAGGG)n, making them the only invertebrates sharing the vertebrate sequence to date. The presence of the vertebrate sequence has been demonstrated in the freshwater clam *Corbicultina leana* (Okazaki et al. 1993) and bay scallop *Argopecten irradians* (Estabrooks 1999) by Southern blot, and in the Pacific oyster *Crassostrea gigas* (Guo & Allen 1997) and the common clam *Donax trunculus* (Gonzáles-Tizón et al. 1998) by fluorescence *in situ* hybridization (FISH). FISH is widely used to assign genes and DNA fragments to chromosomes or subchromosomal regions (Lichter & Cremer 1992). In FISH, DNA fragments are labeled with reporter molecules, such as biotin and digoxigenin, through nick-translation or PCR-incorporation of labeled nucleotides. The labeled probes are denatured and hybridized to denatured metaphase chromosomes. After washing under desired stringencies, hybridized probes are detected with fluorochrome-conjugated reporter binding molecules such as avidin (for biotin) and specific antibodies. Hybridization signals are directly visualized under fluorescence microscope.

To examine if the vertebrate telomeric sequence is shared by a variety of bivalves, we studied the presence and location of this sequence by FISH in four more species: the eastern oyster *Crassostrea virginica* Gmelin, the mangrove oyster *Crassostrea rhizophorae* Guilding, the hard clam *Mercenaria mercenaria* Linnaeus, and the dwarf surfclam *Mulinia lateralis* Say. Here we report the presence of the vertebrate sequence in telomeric regions of all chromosomes in all four species. Our results along with previous studies suggest that the vertebrate telomere sequence, (TTAGGG)n, may be the telomeric sequence for all bivalve molluscs, which at present are the only invertebrates showing the vertebrate lineage in telomeric DNA.

MATERIALS AND METHODS

The eastern oysters used for this study were from a Rutgers University strain, NEH, which has been selected for MSX-resistance for about 40 years and for Dermo-resistance for about 10 years. MSX and Dermo are two diseases caused by the parasites *Haplosporidium nelsoni* and *Perkinsus marinus*, respectively. The mangrove oysters were from a F1 Caribbean population produced and maintained at the Harbor Branch Oceanographic Institute, Florida. The hard clams were obtained from a clam culture company, Biosphere Inc., New Jersey. The dwarf surfclams were collected from beaches of Rhode Island in the fall 2000.

Chromosome Preparation

Metaphase chromosomes were obtained from cultured embryos in all four species, according to the methods described by Guo and Allen (1997). For oysters and the dwarf surfclam, gametes were

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collected by dissecting gonads. For the hard clam, females and males were induced to spawn by thermal simulation.

Eggs were collected by passing through a 60-μm nytex screen to remove large tissue debris and rinsed on a 25-μm screen. Sperm suspension was filtered through a 25-μm screen. Eggs from several (3–6) females and sperm from several males were collected and pooled. Eggs were resuspended in 23–25°C seawater (at about 1000/ml) and fertilized by adding sperm suspension to a final density of about 5–10 sperm per egg. Embryos were cultured in filtered and UV-treated seawater at 23°C. At 6–8 hours post fertilization (about 64-cell stage), embryo suspension was concentrated on a 25-μm screen and harvested into a 15-ml tube: 0.2 ml of embryos per 10 ml seawater. Embryos were treated with 0.005% colchicine in seawater for 10–15 min. Embryos were pelleted by centrifugation at the end of the treatment. Colchicine was replaced by 0.075 M KCl, and the hypotonic treatment lasted for 10–15 minutes. Embryos were then fixed with freshly made Carnoy's fixative, 1:3(v:v) acetic acid and methanol. Fixative was changed twice before storing at 4–7°C.

Chromosome spreads were made by dropping embryo/cell suspension onto clean glass slides and flooding the slides with 2–3 drops of 1:1 methanol and acetic acid. Slides were air-dried at an angle of 45°C. Slides were stored at −20°C until FISH analysis.

In situ Hybridization

FISH was conducted according to a protocol from Oncor Inc, modified and described in Guo and Allen (1997). Prior to hybridization, slides were pretreated in ×2 SSC (0.3 M sodium chloride and 0.03 M sodium citrate, pH = 7.0) at 37°C for 30 minutes. Slides were then dehydrated in 70%, 80% and 95% ethanol for 2 min each and air-dried. Chromosomes on the slides were denatured in 70% formamide in 2× SSC at 72°C for 2 min and dehydrated in a series (70%, 80% and 95%) of ice-cold ethanol. Slides were air-dried, and areas containing chromosome spreads were marked.

A commercially available all-human telomere probe, (TTAGGG)n labeled with digoxigenin and supplied in hybridization solution (Oncor), was used for this study. A 10-μl aliquot of probe was denatured at 72°C for 5 min, immediately cooled on ice and applied to the marked area on each denatured slide. A cover glass was applied and sealed with rubber cement. Slides were then incubated overnight in a humidified chamber at 37°C for hybridization. After hybridization, slides were washed for 5 min in 2× SSC at 72°C, and for three times, 2 min each, in 1× phosphate buffered detergent (PBD, provided by Oncor) at room temperature. The stringency of the post-hybridization wash was increased to 0.5× SSC to test hybridization strength. Negative treatment controls were included and made by replacing the probe with equal amounts of hybridization solution.

Hybridization was detected with fluorescein-labeled anti-digoxigenin antibodies (Ventana). Sixty μl of detection reagent were applied to each slide, covered with a plastic coverslip and incubated at 37°C for 5 min. Detection reagent was washed three times with 1× PBD. Slides were counterstained with 18 μl of propidium iodide/antifade and analyzed under a Nikon epi-fluorescence microscope with PI and PI/FITC dual-pass filters. Metaphase images and FISH signals were captured with a 3CCD camera and analyzed using the Image-Pro Plus software.

RESULTS

Preparations from early embryos provided sufficient metaphases for FISH analysis, and most contained elongated chromosomes. All metaphases analyzed in the eastern and mangrove oysters had 20 chromosomes as expected (Longwell et al. 1967, Rodriguez-Romero et al. 1979). Metaphases from the two clam species had 38 chromosomes as previously reported (Wada et al. 1990, Menzel & Menzel 1965). All chromosomes of dwarf-surf clam are acrocentric.

FISH with human telomeric probe (TTAGGG)n produced strong hybridization signals on interphase nuclei and metaphase chromosomes in all four species examined. Analysis of metaphase chromosomes showed that FISH signals were located at telomeric regions of all chromosomes in all four species: the eastern oyster (Fig. 1A), the mangrove oyster (Fig. 1B), the hard clam (Fig. 1C) and the dwarf surfclam (Fig. 1D). The signals were clear and strong in all four species even under high wash stringency (0.5× SSC). Most images were collected after high stringency wash, although results from low stringency (2× SSC) wash were almost identical, or occasionally stronger. All signals were limited to telomeric regions, and no minor or weak interstitial sites were detected even under low wash stringency.

FISH signals were present at telomeres of two sister-chromatids of each chromosome, although occasionally the signal intensity varied. On some chromosomes, the signal on one chromatid was stronger than that on the other chromatid. In some cases, signal intensity varied somewhat among chromosomes. From several metaphases analyzed, there was no apparent difference in the size or intensity of signals among the four species. No signals were observed in the treatment controls.

DISCUSSION

Results of this study clearly demonstrate that the vertebrate telomeric sequence (TTAGGG)n is present in telomeric regions of all chromosomes in all four bivalve species examined. The strong FISH signals obtained under high stringencies are an indication of complete homology between the probe and targeted sequence, rather than cross-hybridization to related sequences. In a *Corbulina* clam and the bay scallop, the (TTAGGG)n sequence hybridized to genomic DNA in Southern blot, while other telomeric sequences, such as the insect (TTAGG)n, the nematode (TTAGGC)n and the protozoan (TTGGGG)n, did not (Okazaki et al. 1993, Estabrooks 1999). The lack of cross-hybridization among different (although similar) telomeric sequences has also been demonstrated in many other species (Okazaki et al. 1993, Meyne et al. 1995). Other telomeric sequences were not included in this study, because they are not comparable with the vertebrate sequence in length and labeling. Nevertheless, it would be helpful to test all telomeric sequences in molluscs by FISH in the future.

Chromosomal location of the vertebrate sequence has not been studied by FISH in the *Corbiculina* clam and bay scallop. In the Pacific oyster and a *Donax* clam, the vertebrate sequence has been located to telomeric regions of all chromosomes (Guo & Allen 1997, Gonzáles-Tizón et al. 1998). Including the four species examined here, all eight bivalve molluscs studied so far shared the vertebrate telomeric sequence. Those findings lead us to believe that the vertebrate sequence, (TTAGGG)n, may be the telomeric sequence for most, if not, all bivalve molluscs. This finding is interesting, because at present, bivalve molluscs are the only invertebrate group showing the vertebrate lineage in telomeric DNA. Nematodes and most insects have different telomeric sequences (Zakian 1995). However, telomeric sequences in most invertebrates have not been studied, and it is possible that the vertebrate sequence is shared by other invertebrates.

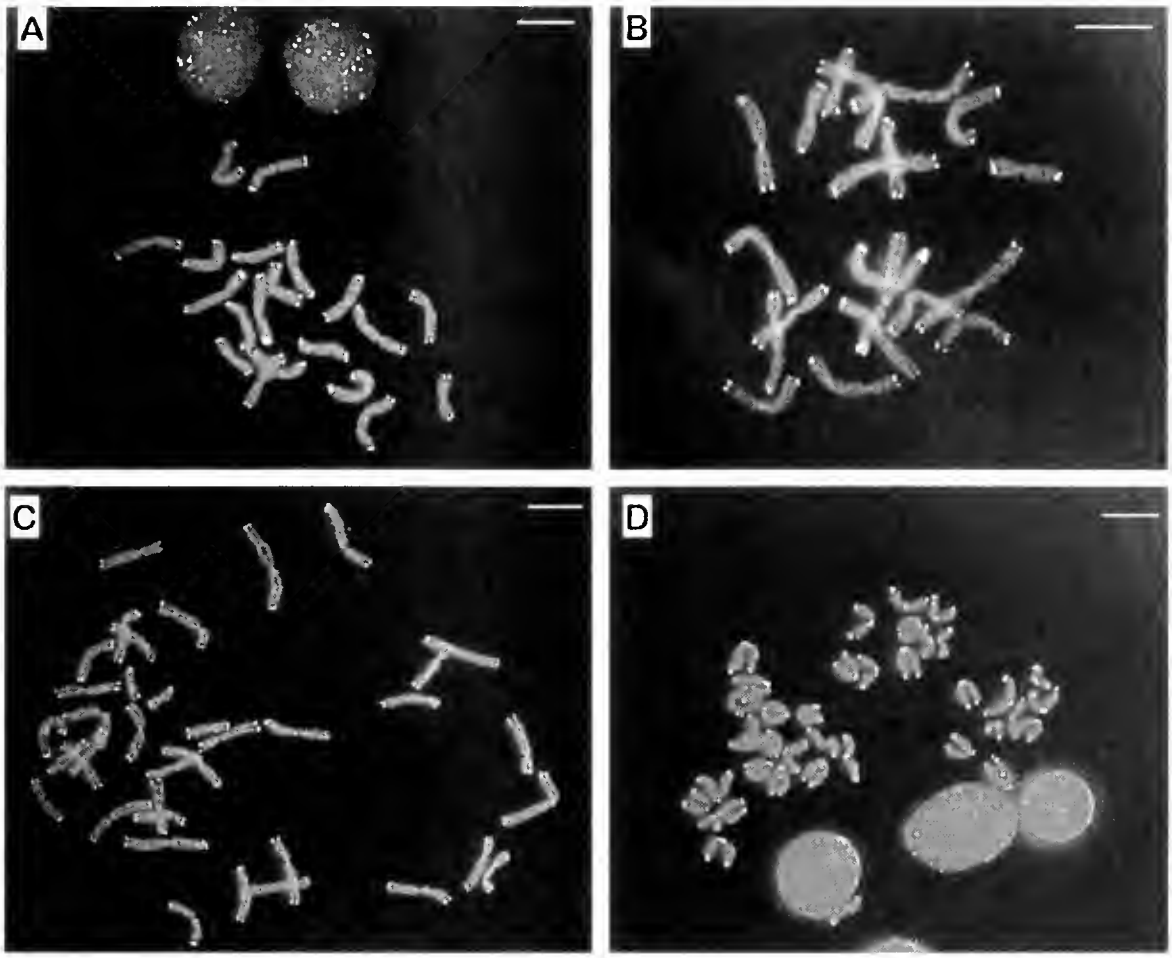

Figure 1. Fluorescence *in situ* hybridization of the vertebrate telomeric sequence (TTAGGG)n to telomeres of all chromosomes in four bivalve molluscs: A, the eastern oyster, *Crassostrea virginica*; B, the mangrove oyster, *Crassostrea rhizophorae*; C, the hard clam, *Mercenaria mercenaria*; and D, the dwarf surfclam, *Mulinia lateralis*. Bar = 5 μm.

It should be pointed out that telomeric DNA in most organisms is more complex than just repeats of one simple sequence. Besides the dominant simple sequence, minor simple repeats and moderate repetitive sequences may occur in telomeric regions (Zakian 1995, Meyne et al. 1995). The complete understanding of bivalve telomeric DNA requires cloning and sequencing of telomeric regions. The vertebrate sequence used in this study can be useful as a probe for cloning studies in bivalves.

Telomeric sequences have been found at interstitial sites in many organisms (Zakian 1995). In this study, all FISH signals were restricted to telomeric regions of chromosomes, and no interstitial sites were detected even under low stringency conditions. No interstitial sites were observed in the Pacific oyster and *Donax* clam (Guo & Allen 1997, Gonzáles-Tizón et al. 1998). The lack of interstitial sites for telomeric sequence may be an indication of limited karyotypic rearrangement through evolution. Karyotypes of *Crassostrea* oysters are highly conserved, and all species studied so far had a diploid number of 20 chromosomes, which are similar in morphology (Nakamura 1985, Xu et al. 2001). It is possible that interstitial sequences are present, but too short to be detected by FISH. The occasionally different signal intensities among some chromosomes and between chromatids may be due to random variations in hybridization condition, or may reflect true differences in target sequence size.

In conclusion, this study provides further evidence that the vertebrate telomeric sequence, (TTAGGG)n, is the telomeric sequence for bivalve molluscs. Studies on additional species are needed to test this hypothesis. It would be interesting to determine if other molluscan groups such as gastropods and cephalopods, also share the vertebrate telomeric sequence. The vertebrate telomeric probe and FISH analysis can be useful in studies on telomeric changes in molluscan development and aging (Harley et al. 1990, Estabrooks 1999).

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ABSTRACTS OF PRESENTATIONS

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CONTENTS

ENHANCEMENT EFFORTS ON STATE TIDELANDS BY THE WDFW INTERTIDAL SHELLFISH ENHANCEMENT PROJECT. Amilee Caffey, Brady Blake, and **Walt Cooke,** Washington Department of Fish and Wildlife, Point Whitney Shellfish Laboratory, Brinnon WA 98320.

Due to an increasing demand for clam and oyster resources on public tidelands in Washington, the Washington Department of Fish and Wildlife (WDFW) developed the Intertidal Shellfish Enhancement Project in 1988. The goal of this project is to increase recreational opportunities by way of planting clam and oyster seed, as well as harvestable size oysters, onto public tidelands. Species targeted in the past have been Pacific oysters, geoduck and Manila clams. In the last few years, research has been conducted on culturing and enhancing native species such as butter, native littleneck clams and cockles. Enhancement efforts occur throughout Puget Sound. Creel surveys and population assessments of targeted clam species are conducted each year by WDFW and tribal staff. This information is passed to the Intertidal Enhancement Project and beaches are seeded according to Intertidal Management plans and needs.

The Enhancement Project purchases Pacific oyster seed and harvestable size oysters to be planted by commercial growers as directed by WDFW staff. The Point Whitney Shellfish Hatchery provides geoduck and Manila clam seed. Research performed by the WDFW Shellfish Hatchery on native species of slams such as butter, littleneck clams and cockles, have provided the Enhancement Project with small groups of seed for test plots, which will, in turn, lead to large scale planting of native species in the future.

THE USE OF HIGH HYDROSTATIC PRESSURE FOR THE REDUCTION OF *VIBRIO PARAHAEMOLYTICUS* IN SHELL OYSTERS. H. Calik, M. T. Morrissey, P. Reno, R. Adams, and **H. An,** Oregon State University Seafood Laboratory, 2001 Marine Drive, Astoria, OR.

High Hydrostatic Pressure (HHP) Technology has shown good potential pathogen reduction. The effect of HHP treatment on pure Vp culture was tested. Clinical and environmental strains of Vp were acquired from FDA was tested. Both clinical and environmental Vp strains were treated with HHP at different settings (1–10 min at 35K psi; 1–5 min at 40K psi; 30–120 s at 45K psi; 10–50 s at 50K psi). Total Vp was enumerated before and after HHP treatment to determine survival.

Additional tests were performed to determine if Vp in shell-oysters responds to HHP treatment differently than Vp in pure culture. After the inoculation of the same clinical and environmental Vp strains, oysters were treated with HHP at the same time and pressure settings.

Results showed that Vp is susceptible to HHP in both pure culture and shell-oyster treatments. The optimum conditions for reducing Vp counts from 109 cfu/ml to 101 cfu/ml was achieved at 50K psi in 30 seconds. At 35K psi, >10 min were required to reduce the count to 101 cfu/ml Vp survival.

HHP treatment was successful in reducing Vp counts. Vp in oysters and pure Vp culture responded similarly to HHP treatments. No resistance variation to HHP was detected between clinical and environmental Vp strains. HHP can be a viable means to reduce the Vp counts in oysters.

THE ROLES OF ENVIRONMENTAL STRESSORS AND CULTURE METHODS ON THE SUMMER MORTALITY OF THE PACIFIC OYSTER *CRASSOSTREA GIGAS*. Daniel Cheney, Ralph Elston, Brian MacDonald, Kendra Kinnan, and **Andy Suhrbier,** Pacific Shellfish Institute, 120 State Ave NE #142, Olympia, WA 98501.

During the late summer to early fall period, Pacific oysters cultured on the west coast of the United States and elsewhere may experience high levels of mortality. In the 1960's to 80's, this condition was subject to intensive investigation focusing on broad areas of disease pathology, genetics, physiology and the environment. Results of these studies were largely inconclusive, or pointed to a poorly defined etiology. Although several factors, such as a bacterial and herpes-like virus infections could be linked to certain mortality events, no clear picture emerged.

Recent studies in Puget Sound, Washington USA and Tomales Bay, California USA center on the influence of multiple stressors and their affects on oyster survival, physiology and pathology. The goal of this research is to identify possible modifications in culture practices, brood stock selection or grow-out location to increase survival of Pacific oysters.

Field observations indicate oysters are subject to extreme variations in a number of parameters during intertidal cycles. Annual or seasonal variations in those parameters and culture practices appear to play a major role in oyster survival. An increased rate of oyster mortality and modified physiological response appears to be strongly correlated with both elevated temperatures and extended periods of depressed DO. A long period of neap tides with low and slack water during the evening was observed to result in daily and successive reductions in DO to levels ranging from 0.5 and 2 mg/L. The DO reductions are sometimes coupled with heavy macroalgae blooms and high phytoplankton densities. This and other work indicate oyster summer mortality rates are also strongly influenced by ploidy and broodstock origin/stock selection. These observations have renewed interest in testing selected stocks with reduced rates of summer mortality, which still retaining desirable characteristics of good growth and meat yield.

IMPLEMENTING A STUDY TO DETERMINE THE VALUE OF MOLLUSCAN SHELLFISH CULTURE AREAS AS FISH HABITAT IN WEST COAST ESTUARIES. **Brett Dumbauld,** Washington State Department of Fish and Wildlife, P.O. Box 190, Ocean Park, WA, 98640, **David Armstrong, Curtis Roegner, Kristine Feldman, Libby Loggerwell**, School of Fisheries, Box 355020, University of Washington, Seattle, Washington 98195, and **Steven Rumrill**, South Slough National Estuarine Research Reserve, P.O. Box 5417, Charleston, Oregon 97420.

The ecological role of bivalve molluscs in estuarine systems has recently been recognized in other areas including Europe and eastern North America where studies have been completed and in some cases shellfish restoration efforts initiated. Comparable studies are lacking however from estuaries on the West Coast, where bivalve (particularly oyster and clam) aquaculture often dominates the intertidal landscape. Due to increasing interest, especially with regard to the potential impacts of oyster aquaculture activities on juvenile salmonids (driven by the listing of several stocks under the Endangered Species Act), we are initiating a study designed to quantify both adverse, but also beneficial impacts of shellfish farming on selected estuarine fauna and flora. We will focus our initial efforts on oyster ground culture and on eelgrass as benthic habitats given the extent and previously documented value of these habitats respectively. Field and laboratory objectives include: 1) utilizing remote sensing and ground-truthing to document annual variability in eelgrass cover in oyster culture and eelgrass meadows in Willapa and Coos Bay estuaries; 2) compare species diversity, density and biomass in culture areas as well as eelgrass meadows; 3) conduct field experiments to examine the impacts of various culture activities on eelgrass and associated infaunal and epifaunal communities; and 4) conduct surveys of fish utilization in oyster beds and eelgrass meadows. Finally, we hope to prepare guidelines to assist both shellfish farmers and estuarine managers in avoiding and/or reducing adverse impacts on estuarine habitat while maximizing the potential beneficial impacts of aquaculture activities.

COMPARISON OF THE FIELD PERFORMANCES OF THE "100% OR NATURAL" TRIPLOID PACIFIC OYSTER *CRASSOSTREA GIGAS* WITH CHEMICAL TRIPLOIDS AND DIPLOIDS. **Benoit Endeline**, Taylor Resources INC, Whiskey Creek Shellfish Hatchery, 2975 Netarts Bay Road W., Tillamook OR 97141.

For about 3 years, the Whiskey Creek Shellfish Hatchery has been producing commercially a new kind of triploid Pacific Oysters (*Crassostrea gigas*) called "Natural" or "100%" triploid, made from the cross between regular diploids and tetraploids. The first advantage is that these "natural" triploids are always 100 percent triploid as verified by flow cytometry, insuring a high quality product for the customers. Hatchery seed was deployed in grow-out bags at two different locations for a 26-month study to examine the growth performance (length, whole weight, meat weight) and gonadal development of the natural triploids, chemical triploids and diploids. The "natural" triploid outperformed both diploids and chemical triploids on most of the characteristics studied. The final length was increased by 7 to 9% in the natural triploids compared to the diploids, the whole weight improved by 30 to 40% and the meat weight by 19% in the most productive area. The "natural" triploids outperformed as well the chemical triploids, with an increase of 7% in length, 15% in whole weight and 18% in meat weight in the most productive area. No histological differences were detected between "natural" and chemical triploids, but their gametogenesis was significantly reduced when compared to the diploids for the females and in a lower extend for the males.

MONITORING THE STATUS OF THE EUROPEAN GREEN CRAB INVASION IN WASHINGTON STATE COASTAL ESTUARIES. **Ronald Figlar-Barnes, Andrea Randall**, and **Brett Dumbauld**, Washington State Department of Fish and Wildlife, P.O. Box 190, Ocean Park, WA, 98640.

The European green crab (*Carcinus maenas*) was discovered on the West Coast of North America in 1989. A series of recruitment events led to expansion northward from San Francisco Bay including a particularly strong recruitment pulse which resulted in numerous crab being found in coastal estuaries from Oregon to British Columbia in 1998 and 1999. Washington State responded by establishing a statewide monitoring and control program for this invader in 1998.

Results from the monitoring effort to date in Willapa Bay and Grays Harbor, Washington suggest that the population in these estuaries is declining, despite evidence that local reproduction is potentially occurring. Average catch per unit trapping effort has declined markedly at a station located near the mouth of Willapa Bay. Mating couples and females with viable eggs were found during winter months and recruitment was documented in 1999, however larger crab representing the 1997/98 year class still dominate the catch. A volunteer trapping effort was successfully instigated along the western shore of Willapa Bay in 2000 and trapping methods continue to be refined as the primary control technique.

BIOLOGY AND FISHERIES POTENTIAL OF THE VARNISH CLAM, *NUTTALLIA OBSCURATA*, IN BRITISH COLUMBIA. **Graham E. Gillespie, Mia Parker,** and **Bill Merilees,** Fisheries and Oceans Canada, Pacific Biological Station, Nanaimo, BC. V9R 5K6.

Varnish clams, *Nuttallia obscurata*, have recently become established in British Columbia (BC), likely from introduction in ballast water. They are currently distributed from Cameleon Harbour (50°22′N) to at least Port Townsend (48°01′N) and on the west coast of Vancouver Island in Barkley and Clayoquot Sounds.

They have been reported from Oregon, but not from Grays Harbour or Willapa Bay, WA.

Varnish clams tend to be distributed at higher tidal elevations than Manila clams, prefer sand or gravel substrates and are often found in areas of freshwater outflows or seepage. When found with Manila clams, varnish clams tend to bury deeper in the substrate. Varnish clams use pedal feeding to augment suspension feeding and this, as well as their tendency to live deep in the substrate, may allow them to live at higher tidal elevations.

Varnish clams spawn in May in the western Pacific and histological examination of a BC sample taken late in March showed evidence that spawning had occurred. Varnish clams appear to grow at similar rates to Manila clams, reaching 38 mm in length in four years.

Varnish clams have potential as a commercial and recreational resources, as attractive clams with good meat-to-shell ratio and good shelf life. They are generally gritty and can recycle purged substrate from tanks. The soft-shelled nature of varnish clams present a problem due to breakage during harvest and transport.

Work is currently underway in BC to explore market potential and develop policy leading to commercial exploitation of varnish clams.

WHERE DOES THE GREEN CRAB FIT INTO THE HIERARCHY OF NATIVE CRAB PREDATORS? Laura Hauck, Sylvia Behrens Yamada, and **Sabre Mahaffy,** Zoology Department, Oregon State University, Corvallis, Oregon 97331-2914.

We compared prey size selection, prey consumption rates and mechanical advantage of the claw lever systems in the European green crab, *Carcinus maenas*, and five species of native crabs. Individual crabs were housed inside perforated plastic boxes in open seawater tanks at Hatfield Marine Science Center. We offered each crab 5 small (5–8 mm) 5 medium (8–11 mm) and 5 large (11–14 mm) snail (*Littorina sitkana*) per day and recorded their consumption rate.

Green crabs eat significantly more snails than the shore crabs *Hemigrapsus nudis* and *H. oregonesis* of similar size. While all 3 crabs prefer smaller snails, only green crabs eat the largest. They simply insert the slender tips of their minor claw into the snail's aperture and pull out the soft tissue.

Green crabs exhibit the same feeding rates as young Dungeness (*Cancer magister*) and red rock crabs (*C. productus*) of similar size. The mechanical advantage of claw lever systems are 0.25 for Dungeness, 0.28 for *Hemigrapsus nudis* and *H. oregonesis*, 0.36 for the master claws of male green crabs and 0.39 for red rock crabs. These comparisons suggest that the impact of green crabs on hard-shelled prey will be significantly higher than that of shore crabs and of the same order of magnitude as that of red rock crabs of similar size. This research was supported by Oregon Sea Grant.

IMPROVEMENTS OF YIELDS OF PACIFIC OYSTERS THROUGH GENETIC SELECTION. Chris Langdon, Dave Jacobson, Sean Matson, and **Ford Evans,** Coastal Oregon marine Experimental Station and Department of Fisheries and Wildlife, Oregon State University, Newport, Oregon, 97365.

Crosses were made in 1998 among nine families showing the highest yields (wet weight per bag) of 48 families planted at an intertidal site in Tomales Bay, California. The resulting full-sib families were plated at an intertidal site in Totten Inlet, Puget Sound, Washington, in 1998. In addition, full-sib families derived from crossing non-selected "wild" oysters were planted at this site as well as groups of oyster seed from various commercial sources. After about two years of growth, the families were harvested and the average yield (kg live weight) per bag determined for each family. The estimated realized heritability for yield was 0.42. Family yields varied greatly depending on the composition of the parental cross, with the average yield of inbred families being significantly less ($p = 0.0028$; Fisher's PLSD) than that of out-bred families from the selected broodstock; however, the average yield of the top nine families from the selected broodstock was 28% greater than that of industry oysters. Based on these results, it should be possible for industry to significantly improve oyster production by crossing specific selected families as part of a long-term breeding program. Inbreeding should be avoided as it results in significantly reduced yields.

SPATIAL STOCK STRUCTURE IN BRISTOL BAY (ALASKA) RED KING CRAB AND ITS INFLUENCE ON LONG-TERM RECRUITMENT TRENDS. Timothy Loher and **D. A. Armstrong,** University of Washington, School of Fisheries, Box 355020, Seattle, WA 98195.

The Bristol Bay red king crab (*Paralithodes camtschaticus*) stock once supported the most lucrative fishery in the world, but low catches over the last ~20 yrs have prompted fishery closures and a host of restrictive regulatory measures. However, these actions have had little effect on stock rebuilding, suggesting that factors outside the fishery may exert strong influence on population abundance.

We hypothesize that the population is regulated by the survivorship of early post-settlement stages that require complex substrates as nurseries, and that low recruitment levels are in part the result of climate forcing that has altered larval source-sink dynamics. We present evidence of alterations in broodstock distribution that occurred during the early 1980's, and changes in spatial recruitment patterns that roughly mimic the shifts in the adult population; the possible effects of the 1978 "climate regime shift" in initiating these changes will be discussed.

INTERTIDAL SPAWNING ECOLOGY OF THREE SPECIES OF MARINE FORAGE FISHES IN WASHINGTON STATE. Daniel E. Penttila, Washington Department of Fish and Wildlife, Marine Resources Div., LaConner, WA 98257.

The Pacific herring (*Clupea*), surf smelt (*Hypomesus*), and Pacific sand lance (*Ammodytes*) are common schooling forage fishes in the Puget Sound basin and coastal estuaries on Washington State. In spite of their pelaigic habitats, they are intimately associated with nearshore/intertidal benthic environments during their annual spawning activity. Herring use nearshore beds of eelgrass and marine algae for spawn deposition. Surf smelt and sand lance use upper intertidal sand-gravel beaches for spawning.

Marine forage fish spawning sites and seasons are unpredictable from year to year. Spawning commonly occurs for several months each year at any given spawning site. Preservation of all known spawning sites is considered critical for these species' long-term conservation.

In many areas, forage fish spawning sites and seasons overlap and coincide with shellfish aquaculture areas and commercial harvest activities. Means must be found to minimize disruption of forage fish spawning sites and seasons. Current environmental regulations consider all known marine forage fish spawning sites to be "habitats of special concern," and they are afforded "no net loss" protection from impacts of in-water human activities. Efforts are being made to inventory all Washington State shorelines for evidence of forage fish spawning activity.

PUGET SOUND DUNGENESS CRAB (*CANCER MAGISTER*) MOLTING PATTERNS. Donald Velasquez and **Steve Burton,** Washington Department of Fish and Wildlife, 16018 Mill Creek Blvd., Mill Creek, WA 98012-1296.

The Washington State Department of Fish and Wildlife and Treaty Tribes currently regulate the harvestable surplus of Dungeness crab in Puget Sound based on the following criteria: 1) male crab only, 2) a minimum size of male crab, and 3) avoiding pot harvest on soft male crab coming out of a major molt. With these conservation tools in place, the harvestable surplus is then allocated between the State and Treaty Tribes for a 50/50 split. State/tribal management plans set pot fishery seasons around the major molts and any deviation from these dates must be accompanied by tests to determine that 80% of the legal-sized, male crabs are in hardshell condition. Data from these shell condition tests indicates that the molting time period once considered appropriate for all of Puget Sound is not applicable for all of it's subareas. The results also suggest a trend in the molt timing from the northern regions to the southern regions.

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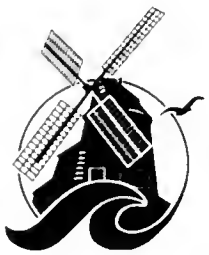

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MONITORING FOR TOXIC CONTAMINANTS IN *MYTILUS EDULIS* FROM NEW HAMPSHIRE AND THE GULF OF MAINE

S. H. JONES,[1]* M. CHASE,[1] J. SOWLES,[2] P. HENNIGAR,[3] N. LANDRY,[4] P. G. WELLS,[3,5] G. C. H. HARDING,[6] C. KRAHFORST,[7] AND G. L. BRUN[8]

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ABSTRACT Gulfwatch is an international monitoring program that uses *Mytilus edulis* as the sentinel species for habitat exposure to toxic contaminants in the Gulf of Maine. Since 1991, the Gulfwatch program has measured the concentrations of 10 trace metals, 17 chlorinated pesticides, 24 polynuclear aromatic hydrocarbons (PAHs), and 24 polychlorinated biphenyl (PCB) congeners in mussel tissue from over 60 sites throughout the Gulf of Maine. In 1998, the Gulfwatch program included a more intensive survey of sites in New Hampshire. Of the 21 sites sampled in the Gulf in 1998, six sites were sampled in New Hampshire and the other 15 sites were in Nova Scotia, New Brunswick, Maine, and Massachusetts. Results for the Gulfwide program showed a southward trend of increasing concentrations for organic contaminants and Ag, Cr, and Pb, reflecting major local and regional pollution sources. The distribution of the remaining trace metals was more uniform, without obvious impacts from the more heavily urbanized areas of the southern gulf. At some sites there appears to be localized hot spots for a variety of contaminants. Gulfwide geometric mean (GM) concentrations were lower than GM +85% confidence level (CL) concentration for the NOAA National Status and Trends (NS&T) Mussel Watch program, except for Hg. Two sites had Pb concentrations that exceeded U.S. FDA guideline levels. While Hg concentrations did not exceed the U.S. FDA action concentration at any site, the NS&T GM +85% CL was exceeded at all 1998 Gulfwatch sites. The spatially intensive New Hampshire program has been useful in identifying local sources of contaminants and determining the significance of mussel exposure to oil from chronic discharges and larger spills. Similar to comparisons between Gulfwatch and NS&T data, interpretation of New Hampshire results benefit from comparisons to the greater Gulfwatch program. The Gulfwatch database provides a useful regional perspective to interpret the results from the New Hampshire program and for other localized studies. Gulfwatch provides unique, Gulfwide status and trend information and helps to focus efforts to reduce loading of contaminants such as Hg.

KEY WORDS: *Mytilus edulis*, toxic contaminants, Gulf of Maine, spatial trends

INTRODUCTION

The Gulf of Maine (GOM) extends from Cape Sable, Nova Scotia, through New Brunswick, Maine, and New Hampshire to Cape Cod, Massachusetts, and includes the Bay of Fundy and Georges Bank. The ecosystem is one of the world's most productive systems and supports a vast array of species, including some of great commercial importance. Commercial fisheries, including aquaculture, is its principal income-generating enterprise. Tourism is also a significant source of income to GOM coastal communities. Increases in coastal populations and industrial and residential development have contributed to the deteriorating quality of sections of the Gulf's coastal environment (Crawford & Sowles 1992, Dow & Braasch 1996). Despite efforts to improve pollution treatment, the result of increased human growth and activities is the steady input of toxic chemicals into estuarine and coastal environments. Many human-made chemicals can be bioaccumulated to concentrations significantly above ambient levels. Furthermore, some of these environmental contaminants may also be present at concentrations considered toxic to organisms, and thus induce adverse biological effects on productivity, reproduction, and survival of marine organisms and humans (Kawaguchi et al. 1999, Wells & Rolston 1991).

Monitoring programs may use a variety of organisms as indicators of environmental contamination. One organism that has been commonly employed and has proven useful for biomonitoring chemical contamination is *Mytilus edulis* L. (Sericano et al. 1995, Tripp & Farrington 1985). *M. edulis* and other bivalves have been used throughout the world as a measure of the spatial and temporal trends in habitat exposure to chemical contaminants. In addition, *M. edulis* has been successfully used as an indicator organism in environmental monitoring programs throughout the world to identify variation in chemical contaminants between sites, contributing to the understanding of trends in coastal contamination (NAS 1980, NOAA 1991, O'Connor 1992, O'Connor & Beliaeff 1995, Widdows et al. 1995, Cantillo 1998). The blue mussel is the indicator organism for the NOAA NS&T, Gulf of Maine Gulfwatch and New Hampshire Gulfwatch programs because of the following favorable characteristics: mussels are indigenous and abundant in many coastal areas, and are easy to collect and process; much is known about mussel biology and physiology; they are a commercially important food source; mussel contamination is a public health concern; and they are sedentary suspension-feeders that concentrate chemicals in tissue, making measurement of chemicals in mussel tissue an assessment of local biologically available contamination.

The overall objective of this paper is to introduce the Gulfwatch program to a wider audience of scientists concerned about the safe consumption of molluscan shellfish. The 1998 results are

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presented to illustrate local, regional, and international implications and to address issues related to toxic contaminant pollution in the marine environment. Between 1991 and 1997 the Gulfwatch program measured the concentrations of trace metals, polynuclear aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and chlorinated pesticides in mussel tissue at 56 sites throughout the GOM. In 1998, the Gulfwatch program was in the sixth year of a nine-year monitoring plan. By design of the Gulfwatch program, the 1998 sampling was the second sampling of sites previously sampled in 1995. These sites will be resampled in 2001 (Jones et al. 1998). Native mussels collected at 21 sites throughout the Gulf of Maine were analyzed for organic and inorganic contaminants (Crawford & Sowles 1992) to build on the program's Gulfwide assessment of long-term exposure to bioavailable contaminants. One location in each of the five jurisdictions served as a baseline station that has been resampled every year. The 1998 Gulfwatch program also included more spatially intensive sampling in New Brunswick.

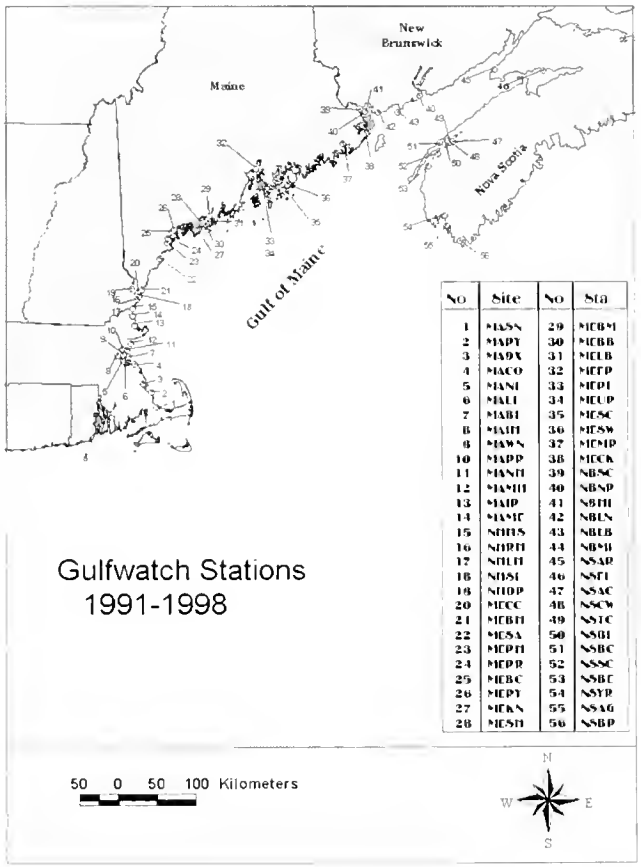
MATERIALS AND METHODS

The 1998 Gulfwatch sample collection and analysis is the sixth year of the program's nine-year sampling design (Sowles et al. 1997). The 1998 sampling represents the third year of the second three-year cycle. As such, a subset of the total Gulfwide stations sampled in 1998 were previously sampled in 1995. Some select sites have been sampled each year since 1993 for more intensive temporal analysis of contaminant concentrations. The New Hampshire Gulfwatch program added six new sites in addition to six sites previously sampled under the Gulfwide Gulfwatch program; 1998 is the first year of sampling at three of the new sites in New Hampshire.

Locations for sites sampled as part of the Gulfwatch program prior to 1998 are shown in Figure 1. The stations sampled in 1998 are presented in Table 1 with reference to site numbers in Figure 1. The site list includes two New Brunswick sites located in St. John Harbor that were new in 1998. The six New Hampshire sites sampled in 1998 are shown in Figure 2, including three sites that had been previously sampled, NHLH, NHDP, and MECC, and three sites that were sampled for the first time: NHGP, NHSS, and NHNM. Clark Cove (MECC) in Portsmouth Harbor is a benchmark site that has been sampled each of the previous five years. Gulfwatch benchmark sites were established to enable trend analysis and were selected as sites considered to be important in each jurisdiction.

Field and Laboratory Procedures

Details regarding the mussel collection, measurement, and sample preparation are published in Sowles et al. (1997). All field sampling was conducted in the fall of 1998. No sampling occurred during or shortly after periods when storm water runoff and wave resuspension of bottom sediment could result in enhanced uptake and accumulation of sediment in the mussel gut, as previously reported (Chase et al. 1998). Mussels were collected from four discrete areas within a segment of the shoreline that is representative of local water quality. Using a wooden gauge or a ruler, 45–50 mussels of 50–60 mm shell length were collected. The mussels were cleaned of all sediment, epibiota, and other accretions in clean seawater from the collection site, placed in clean containers, then transported to the lab in coolers with ice packs. Mussels were not depurated prior to processing. Prior to shucking, mussels were thoroughly washed to minimize tissue contamination from any remaining surface debris.



No	Site	No	Sta
1	MASS	29	MEBM
2	MAPY	30	MEBB
3	MAPR	31	MELB
4	MACO	32	MEFP
5	MANI	33	MEPI
6	MALI	34	MEUP
7	MABI	35	MESC
8	MAIH	36	MESW
9	MAWS	37	MEMP
10	MAPP	38	MECK
11	MANH	39	NBSC
12	MAMH	40	NBNP
13	MAIP	41	NBTH
14	MAMC	42	NBLN
15	NHHS	43	NBLB
16	NHRH	44	NBMH
17	NHLH	45	NSAP
18	NHSI	46	NSFI
19	NHDP	47	NSAC
20	MECC	48	NSCW
21	MEBH	49	NSTC
22	MESA	50	NSBI
23	MEPH	51	NSBC
24	MEPR	52	NSSC
25	MEBC	53	NSBE
26	MEPT	54	NSYP
27	MEKN	55	NSAG
28	MESH	56	NSBP

Figure 1. Location of Gulfwatch stations in the Gulf of Maine.

In the laboratory, mussels were shucked directly into appropriately prepared Mason jars for metal and organic analysis, respectively (for details see Sowles et al. 1997). Composite samples (20 mussels/composite; four composites/station) were capped, labeled, and stored at −15° C for three to six months prior to analysis.

Analytical Procedures

Analytical procedures followed those reported for the previous years (Chase et al. 1998, Jones et al. 1998). Table 2 contains a summary of measured trace metal and organic compounds. Inorganic contaminants were analyzed at the State of Maine Health and Environmental Testing Laboratory (Augusta, ME). Analyses for mercury were done on a subsample of 1 to 2 g of wet tissue and measured by cold vapor atomic absorption on a Perkin Elmer Model 503 atomic absorption spectrometer. Analyses for all other metals were conducted on 5 to 10 g of wet tissue dried at 100°C. Zinc and iron were measured by flame atomic absorption using a Perkin Elmer Model 1100 atomic absorption spectrometer. All remaining metals (Ag, Al, Cd, Cr, Cu, Ni, and Pb) were analyzed using Zeeman background-corrected graphite furnace atomic absorption with a Varian Spectra AA 400. The analyte detection limits for the metals in μg/g dry weight (DW) are as follows: Ag, 0.1; Al, 4.0; Cd, 0.1; Cr, 0.2; Cu, 0.4; Fe, 4.0; Hg, 0.1; Ni, 0.4; Pb, 0.4; and Zn, 0.5.

Organic contaminants in mussel samples were analyzed at the Environment Canada, ECB Laboratory in Moncton, NB. The ana-

TABLE 1.

Gulf of Maine Gulfwatch study site locations in 1998.

Station	Map # (Figure 1)	Station Code
Massachusetts		
Sandwich	1	MASN
Boston Inner Harbor	9	MAIH
Pines River	11	MAPR
New Hampshire		
Little Harbor	18	NHLH
Gypsum Plant	Portsmouth Harbor	NHGP
North Mill Pond	Portsmouth Harbor	NHNM
Schiller Station	Portsmouth Harbor	NHSS
Dover Point	20	NHDP
Clark Cove	21	MECC
Maine		
Kennebec River	28	MEKN
Damariscotta	32	MEDM
Boothbay Harbor	30	MEBB
New Brunswick		
Niger Reef	41	NBNR
Chamcook	42	NBCH
Letang Estuary	43	NBLN
Limekiln Bay	44	NBLB
Tin Can Beach	St. John Harbor	NBTC
Coast Guard Wharf	St. John Harbor	NBCG
Nova Scotia		
Cornwallis	50	NSCW
Digby	52	NSDI
Broad Cove	53	NSBC

lyte detection limits ranged from 3.0 to 10 ng/g for aromatic hydrocarbons, from <1.0 to 2.0 ng/g for PCB congeners, and from <1.0 to 2.0 ng/g for chlorinated pesticides. Eighteen of the PCB congeners identified and quantified correspond to congeners analyzed by the National Oceanographic and Atmospheric Administration's (NOAA) National Status and Trends (NS&T) Program designated congeners. Other organic compounds selected for analysis are also generally consistent with NOAA National Status and Trends mussel monitoring (NOAA 1989).

A description of the full analytical protocol and accompanying performance-based QA/QC procedures are found in Sowles et al. (1997), and more comprehensively in Jones et al. (1998). Tissue samples were extracted by homogenization with an organic solvent and a drying agent. Solvent extracts were obtained by vacuum filtration, and biomatrix interference was separated from target analytes in extracts by size exclusion chromatography. Purified extracts were subjected to silica gel liquid chromatography, which provided a nonpolar PCB/chlorinated pesticides fraction and a polar chlorinated pesticide fraction. PCBs and pesticides were analyzed by high-resolution dual column gas chromatography/electron capture detection (HRGC/ECD). Following PCB and pesticide analysis, the two fractions were combined and the resulting extract was analyzed for aromatic hydrocarbons by high-resolution gas chromatography/mass spectrometry (HRGC/MS).

Standard laboratory procedures for metals incorporated method blanks, spike matrix samples, duplicate samples, surrogate addition, and standard oyster tissue (SRM 1566A). The method blanks were inserted as follows: three at the beginning of the run, one at the end, and six at various intervals during the run. Duplicate samples and matrix spike recoveries were conducted on 15% of the samples. The Moncton laboratory participated in the NIST Status and Trends Intercomparison Marine Sediment Exercise IV and Bivalve Homogenate Exercise. Internal quality control and method performance specifications are described in the Environment Canada Shellfish Surveillance Protocol (Sowles et al. 1997, Jones et al. 1998). The protocol includes mandatory QC measures with every sample batch including method blanks, spike matrix samples, duplicate samples, surrogate addition, and certified reference materials (SRM 1974a). The protocol specifies the performance criteria relevant to method accuracy, precision, and detection limits and data reporting requirements for the analysis of organic contaminants in shellfish samples.

Data Analysis

Total PAH ($\sum PAH_{24}$), total PCB ($\sum PCB_{24}$), and total pesticides ($\sum TPEST_{17}$) values were calculated from the sum of all individual compounds or congeners with values greater than the detection limit for the compound. Total DDT ($\sum DDT_6$) is the sum of o,p'-DDT and p,p'-DDT and homologues (o,p'-DDE, p,p'-DDE, o,p'-DDD, and p,p'-DDD). Several tissue samples for metals and organics were below the detection level. Variables in which all replicate measurements were below the detection limit were treated as zero and recorded as not detected (ND). However, if at least one of the replicates was greater than the detection limit, then the other replicates were recorded as 1/2 the detection limit.

All metal data, with the exception of Ag and Ni, were $\log_{10}$ transformed to correct for heterogeneity of variances, whereas all organic contaminant, Ag, and Ni data were $\log_{10}(x + 1)$ trans-

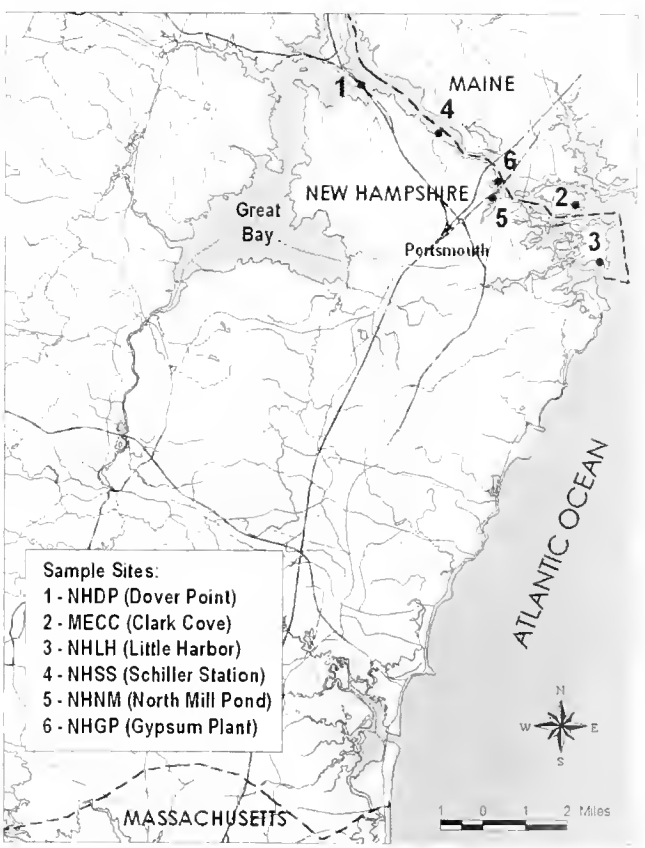


Figure 2. Location of 1998 Gulfwatch stations in New Hampshire.

formed. At each site, arithmetic means were used to summarize the results of replicate samples and are used in all subsequent tables and figures. In addition, geometric means were calculated for each metal and organic contaminant for comparison with other data sets. The confidence levels (CL) around the geometric mean were calculated as:

TABLE 2.

Inorganic and organic contaminants analyzed in mussel tissues from the seacoast of New Hampshire in 1998.

Inorganic Contaminants	Organic Contaminants			
Metals	Aromatic hydrocarbons			
Ag	Naphthalene			
Al	1-Methylnaphthalene			
Cd	2-Methynaphthalene			
Cr	Biphenyl			
Cu	2,6-Dimethylnaphthalene			
Fe	Acenaphthylene			
Hg	Acenaphthene			
Ni	2,3,5-Trimethylnaphthalene			
Pb	Fluorene			
Zn	Phenanthrene			
	Anthracene			
	1-Methylphenanthrene			
	Flouranthene			
	Pyrene			
	Benzo[*a*]anthracene			
	Chrysene			
	Benzo[*b*]flouranthrene			
	Benzo[*k*]flouranthrene			
	Benzo[*a*]pyrene			
	Benzo[*e*]pyrene			
	Perylene			
	Indeno[1,2,3-*cd*]pyrene			
	Dibenzo[*a,h*]anthracene			
	Benzo[*g,h,i*]perylene			
	Chlorinated Pesticides			
	Hexachlorobenzene (HCB)			
	gamma-hexachlorocyclohexane (HCH)			
	Heptachlor			
	Heptachlor epoxide			
	Aldrin			
	Mirex			
	cis-Chlordane			
	trans-Nonachlor			
	Dieldrin			
	Alpha-Endosulfan			
	beta-Endosulfan			
	DDT and homologues			
	2,4′-DDE			
	2,4′-DDD			
	2,4′-DDT			
	4,4′-DDE			
	4,4′-DDD			
	4,4′-DDT			
	PCB congeners			
	PCB 8	PCB 18	PCB 28	PCB 29
	PCB 44	PCB 50	PCB 52	PCB 66
	PCB 77	PCB 87	PCB 101	PCB 105
	PCB 118	PCB 126	PCB 128	PCB 138
	PCB 153	PCB 169	PCB 170	PCB 180
	PCB 187	PCB 195	PCB 206	PCB 209

$$L_1 = \text{antilog}[(\text{mean of } \log Y) - \dagger_{.05[n-1]}\text{SQRT}(s^2_{\log y}/n)] \quad (1)$$

and

$$L_2 = \text{antilog}[(\text{mean of } \log Y) + \dagger_{.05[n-1]}\text{SQRT}(s^2_{\log y}/n)] \quad (2)$$

s^2 = sample variance

RESULTS AND DISCUSSION

Trace Metal Contaminants

Table 3 contains the metal concentrations (arithmetic mean ± SD, μg/g DW) for mussels from all site composite ($n = 4$) samples in 1998. Overall metal concentrations for indigenous mussels are given as geometric means and CI range (Table 3). Trace metals were detected at all sites except for Ag, which was below the detection limit (0.1 μg/g DW) at 10 of the 21 sites. No site had extremely high concentrations of any trace metal, although some sites like MAIH and most of the New Brunswick sites had elevated concentrations of some of the metals. Table 3 includes values for geometric means (GM) and GM +85% CL from the 1990 NS&T Mussel Watch data (O'Connor & Beliaeff 1995) for comparison. Using the NS&T GM +85%CL value as a reference for comparative purposes, two sites exceeded the Ag value, four sites exceeded the Cr value, six sites exceeded the Cu value, 17 sites exceeded the Hg value, two sites exceeded the Ni value, and six sites exceeded the Pb value out of the total of 21 sites. This suggests localized sources of these contaminants at those sites. However, for Cr, Cu, Pb, and especially Hg, more widespread elevated levels suggest possible regional sources of these contaminants.

The tissue analysis for Al and Fe is included to serve as an indication of the degree to which sediment contamination may be present in mussel tissue (i.e., the gut). The fact that four of the six New Brunswick sites had relatively high concentrations of Al and Fe suggests that the mussel tissue samples contained elevated levels of sediment. The presence of sediment in the mussels was suspected in samples having elevated concentrations of some metals (iron, aluminum, and associated metals) (Lobel et al. 1991, Robinson et al. 1993). Sites in the Bay of Fundy are dominated by extensive intertidal mudflats that can be subject to considerable resuspension during windy storm events.

The concentrations of most metals were relatively evenly distributed around the Gulf of Maine (Table 3). There were no apparent spatial trends; however, an occasional hot spot of elevated concentrations was observed. Figure 3 shows the concentrations of Ag, Pb, and Hg measured in the tissue of *M. edulis* at the 1998 sampling stations. The order in which sites are presented is from Cape Cod Bay, up along the coasts of New Hampshire and Maine, into the Bay of Fundy, and along the western shore of Nova Scotia.

Silver concentrations ranged from below the detection limit (0.1 μg/g DW) to 1.82 ± 0.20 (NBCG) and showed strong geographical hot spots of elevated concentrations in areas in each jurisdiction except New Hampshire along the Gulf of Maine (Fig. 3). The highest concentrations were observed in Massachusetts from Boston Harbor south to Sandwich and in New Brunswick around St. John Harbor. Elevated silver exposure concentrations have been shown to coincide with regions receiving municipal sewage (Sanudo-Wilhelmy & Flegal 1992, ten Brink et al. 1996). Because of silver's use in the photographic and jewelry industries, the coastal waters of Massachusetts are up to 1,000 times more concentrated in Ag than in other Gulf of Maine waters (Krahforst & Wallace 1996). The high levels observed at MASN, which is in an area with no known significant source of municipal waste, may

TABLE 3.

Tissue metal concentrations (μg/g dry weight, mean ± SD) for Gulfwatch mussels in 1998.

Station	Ag	Al	Cd	Cr	Cu	Fe	Hg	Ni	Pb	Zn
MASN	0.93 ± 0.40	70 ± 20	1.93 ± 0.49	1.13 ± 0.23	6.37 ± 1.52	217 ± 47	0.36 ± 0.06	0.97	3.23 ± 0.75	15.6 ± 0.1
MAIH	0.15 ± 0.13	155 ± 34	2.65 ± 0.34	2.23 ± 0.80	17.75 ± 7.68	502 ± 123	0.55 ± 0.01	1.35 ± 0.10	26 ± 13	14.6 ± 0.5
MAPR	ND	167 ± 60	1.80 ± 0.22	2.35 ± 0.91	8.15 ± 1.67	322 ± 78	0.48 ± 0.07	1.43 ± 0.21	5.65 ± 1.84	14.8 ± 0.3
NHDP	ND	202 ± 39	2.80 ± 0.28	2.95 ± 0.06	6.06 ± 0.69	385 ± 50	0.97 ± 0.05	1.70 ± 0.20	3.02 ± 0.31	130 ± 14
NHGP	ND	175 ± 49	1.92 ± 0.52	2.08 ± 0.56	4.70 ± 1.27	358 ± 103	0.86 ± 0.09	1.35 ± 0.24	3.32 ± 0.56	111 ± 25
NHLH	ND	162 ± 31	2.42 ± 0.10	2.75 ± 0.97	5.12 ± 0.33	400 ± 45	1.00 ± 0.05	1.72 ± 0.17	4.65 ± 0.37	105 ± 17
NHNM	ND	260 ± 54	1.98 ± 0.37	2.32 ± 0.43	6.55 ± 0.60	482 ± 99	0.79 ± 0.12	1.24 ± 0.20	5.18 ± 1.45	135 ± 21
NHSS	ND	192 ± 34	2.25 ± 0.51	2.30 ± 0.18	6.12 ± 0.49	385 ± 38	1.08 ± 0.10	1.45 ± 0.24	3.15 ± 0.48	128 ± 10
MECC	ND	298 ± 64	2.08 ± 0.13	3.18 ± 0.69	7.20 ± 0.67	528 ± 80	0.82 ± 0.11	2.32 ± 1.08	5.75 ± 0.70	135 ± 24
MEKN	0.10 ± 0.04	117 ± 26	2.08 ± 0.43	1.27 ± 0.23	5.33 ± 0.62	225 ± 42	0.41 ± 0.09	1.02 ± 0.12	1.58 ± 0.40	53 ± 9.9
MEDM	0.15 ± 0.06	292 ± 94	1.23 ± 0.10	1.25 ± 0.21	5.23 ± 0.51	345 ± 101	0.34 ± 0.10	1.03 ± 0.15	1.75 ± 0.19	67 ± 3.5
MEBB	0.07 ± 0.03	258 ± 103	0.96 ± 0.10	1.25 ± 0.26	13.5 ± 1.9	380 ± 109	0.52 ± 0.04	0.90 ± 0.14	15.8 ± 2.1	111 ± 13
NBNR	ND	285 ± 45	0.74 ± 0.05	0.82 ± 0.11	4.58 ± 0.39	358 ± 43	0.22 ± 0.03	0.96 ± 0.05	0.58 ± 0.12	65 ± 3.4
NBCH	ND	175 ± 21	0.88 ± 0.17	0.69 ± 0.06	5.15 ± 0.47	245 ± 24	0.22 ± 0.05	0.87 ± 0.08	0.58 ± 0.17	68 ± 8
NBLN	0.05 ± 0.07	777 ± 148	1.50 ± 0.10	17.5 ± 31.5	12 ± 4	678 ± 315	0.11 ± 0.00	9.8 ± 17.4	1.6 ± 0.4	82 ± 8
NBLB	0.04 ± 0.03	835 ± 53	1.50 ± 0.10	2.5 ± 0.5	13 ± 2	609 ± 61	0.14 ± 0.03	1.4 ± 0.1	2.7 ± 0.1	85 ± 11
NBTC	0.16 ± 0.13	2925 ± 1870	2.50 ± 0.30	12.9 ± 7.8	29 ± 8	2131 ± 764	0.33 ± 0.03	6.6 ± 3.3	2.3 ± 0.8	110 ± 24
NBCG	1.82 ± 0.20	793 ± 179	2.00 ± 0.30	4.0 ± 1.0	29 ± 10	696 ± 160	0.29 ± 0.02	1.9 ± 0.4	2.3 ± 0.3	139 ± 10
NSBC	ND	180 ± 35	1.70 ± 0.00	1.33 ± 0.12	6.73 ± 0.21	313 ± 46	0.37 ± 0.03	1.20 ± 0.10	1.67 ± 0.15	84 ± 8
NSCW	0.08 ± 0.05	388 ± 46	2.73 ± 0.39	1.70 ± 0.14	5.70 ± 1.15	522 ± 59	0.45 ± 0.07	1.88 ± 0.26	3.40 ± 0.39	87 ± 21
NSDI	0.08 ± 0.05	338 ± 31	1.60 ± 0.18	1.43 ± 0.22	5.33 ± 1.44	478 ± 39	0.46 ± 0.06	1.63 ± 0.15	2.70 ± 0.22	94 ± 16
Geometric mean	0.11	270	1.63	1.78	7.61	416	0.42	1.27	2.63	96
LCL to UCL	0.03 to 0.41	141 to 517	1.11 to 2.4	0.96 to 3.29	4.62 to 12.6	271 to 638	0.24 to 0.76	0.84 to 1.91	1.31 to 5.29	69 to 134
NS&T Mussel Watch 1990 data (O'Connor & Beliaeff 1995)										
Geometric mean	0.17	none	2.8	1.7	8.9	none	0.09	none	1.9	130
GM +1 SD	0.58	none	5.7	3	11	none	0.24	3.3	4.3	190

The geometric mean and CL for all mussels are given; $n = 4$ replicates per site
ND = not detected

be a function of transport and deposition of sewage-derived particles (Bothner et al. 1993) that are sequestered in Cape Cod Bay and accumulated by mussels.

The concentration of lead ranged from a value of 0.58 ± 0.12 μg/g DW at two New Brunswick sites (NBNR and NBCH) to 26 ± 13 μg/g DW at Boston Inner Harbor (MAIH) (Table 3, Fig. 3). Lead levels at Boothbay Harbor, ME (MEBB) were also much higher than most other sites. Sediment particles containing Pb may be transported to Boothbay Harbor from the Kennebec-Androscoggin watershed (Larsen & Gaudette 1995). Lead concentrations were generally higher in the southwestern areas compared to the northern and eastern sites (Fig. 3). Mean concentrations of Pb in mussels from coastal regions generally range from 1 to 16 μg/g DW (Fowler 1990). All six of the NH sites sampled in 1998 exceed the Maine reference concentration (ME-RM; Sowles 1993) of 2.6 ± 1.1 μg/g DW, but no site exceeded the Maine high value (ME-HV) (6.00 μg/g DW). MAIH is located in a highly urbanized harbor and subjected to heavy industry, marine transport activities, and municipal waste discharges. Elevated lead in the New Hampshire sites may be related to the close proximity of the sites to the Portsmouth Naval Shipyard where waste plating sludge and lead batteries, respectively, were disposed and stored (NCCOSC 1997). The potential for the shipyard to be a source of lead to estuarine biota was demonstrated in July 1999 when significant amounts of soil from a contaminated site at the Shipyard, containing as much as 14.2 mg Pb/g soil DW, was discovered to be eroding into the Piscataqua River (Cohen 2000). Mussels collected from sites in close proximity to the eroding soil contained Pb concentrations as high as 199 μg/g DW.

The concentration of mercury in mussel tissue ranged from a value of 0.11 μg/g DW at NBLN to 1.08 ± 0.10 μg/g DW at NHSS (Table 3, Fig. 3). Mercury exceeded the NS&T GM +85% CL of 0.24 μg/g DW at 17 of the 21 sites. The New Hampshire sites are markedly higher than sites in other jurisdictions. There are several known historical mercury sources in the New Hampshire Seacoast, including some that are suspected to be related to the Portsmouth Naval Shipyard (NCCOSC 1997) and, especially, the PSNH Schiller Station (NHSS) on the Piscataqua River, where mercury steam was used from 1950 to 1968 (Nelson 1986). Analysis of the mussel tissue concentrations of Hg revealed that there was a significant difference in Hg concentrations between NHSS and all other New Hampshire sites except NHLH. Mean values of Hg in *Mytilus* spp. from coastal regions worldwide range from 0.1 to 0.4 μg/g DW (Kennish 1997), but can be much higher in areas like the southwest Pacific, where sites average as much as 2.7 μg Hg/g DW (Fowler 1990). In a review of the first five years of the Gulfwatch program, tissue concentrations of Hg were discussed as being unusually high and a possible concern (Jones et al. 1998).

Recent studies have shown that a mercury problem exists in freshwater systems of the northeast United States and maritime provinces of Canada (Welch 1994, DiFranco et al. 1995, Evers et al. 1996). About 47% of mercury deposition in the region originates from sources within the region, 30% originates from U.S. sources outside the region, and 23% originates from the global atmospheric reservoir (NESCAUM 1998). On June 8, 1998, the New England governors and eastern Canadian premiers agreed to cut regional mercury emissions from power plants, incinerators, and other sources in half by the year 2003. However, until recently few large coastal systems have been known to be impacted by Hg pollution. Atmospheric mercury deposition measurements made at New Castle, NH, at the mouth of Portsmouth Harbor, showed ~8 ng/m^2 total mercury was deposited during 1998 (MDN, unpub-

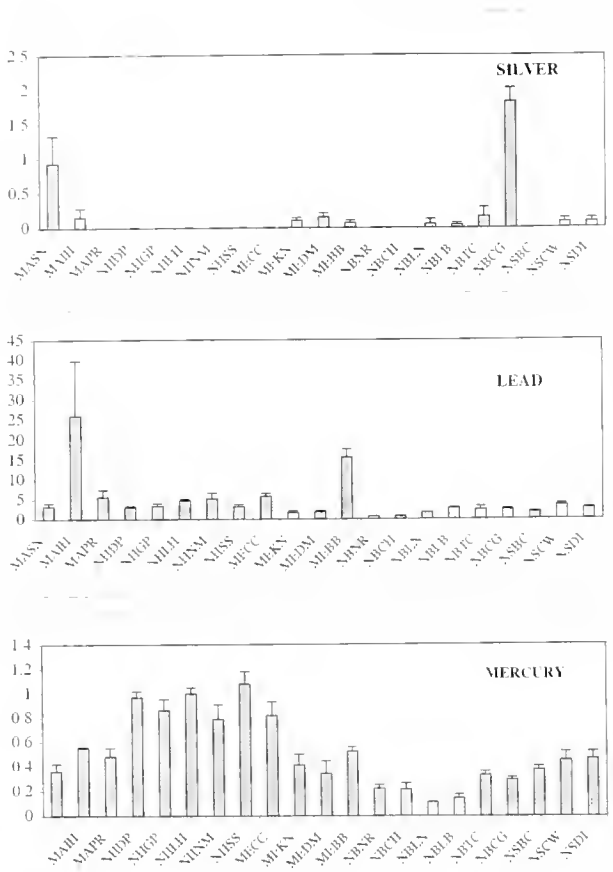


Figure 3. Distribution of silver, lead, and mercury tissue concentrations (arithmetic mean ± SD, µg/g dry weight) in mussels at all Gulfwatch stations in 1998.

lished). The New Castle site, along with two other Maine coastal sites in Casco Bay and Acadia National Park, showed somewhat elevated total mercury atmospheric deposition compared to nearby, upstream inland sites. Other areas in the Gulf of Maine have elevated (5–20 µg/g) sediment mercury concentrations (ten Brink et al. 1997), including the Penobscot River near Orrington, where permitted and accidental discharges from the Holtra-Chem facility have resulted in sediments having much higher (>100 µg/g) Hg concentrations (MEDEP, unpublished). Thus, data on mussel tissue mercury levels may have added importance in assessing current contamination problems and the effects of discharge reduction efforts related to Hg in the future.

Organic Contaminants

The total concentration of detectable polynuclear aromatic hydrocarbons (ΣPAH_{24}), polychlorinated biphenyls (ΣPCB_{24}), and organochlorine pesticides ($\Sigma TPEST_{17}$) measured in mussel tissue samples of indigenous mussels are presented in Table 4. Individual analyte concentrations of each compound class are not shown. In 1998, as in previous reports (Sowles et al. 1996, Chase et al. 1998, Jones et al. 1998), ΣDDT_6 and its degenerative metabolites were the main contributors to total detectable pesticides (data not shown). Analytes within each category of organic contaminant were detected at most sites, except for ΣPAH_{24} at MEDM and ΣPCB_{24} at NBCH, NSBC, and NSCW. There were much wider ranges in concentrations of organic compared to trace metal contaminants. None of the Gulfwatch sites had organic contaminant concentrations that exceeded any human health criteria. For example, the U.S. FDA Action Limit for PCB is 2 µg/g DW (USFDA 1990), and the concentration for the highest Gulfwatch site was 0.74 µg/g DW at MAIH, which is well below this limit.

A clear pattern of higher concentrations in the southwestern Gulf compared to the northeastern Gulf is apparent for all three types of organic contaminants (Table 4). This pattern can be seen in Figure 4, which shows the concentrations of ΣPAH_{24} and ΣPCB_{24} measured in *M. edulis* tissue from the 1998 sites, presented from south to north. The mean of the ΣPAH_{24} concentrations ranged from not detected (ND) at MEDM to 3,330 ng/g DW at MAIH. Mean concentrations of ΣPAH_{24} at all but a few of the Maine and New Brunswick sites were as high as those reported from areas influenced by oil spills and municipal sewage outfall (148 ng/g in Rainio et al. 1986, 63–1,060 ng/g in Kveseth et al. 1982). However, only samples from the Boston Harbor station (MAIH) had concentrations as high as in industrialized areas affected by coking operations, as documented in Sydney Harbor, NS (1,400–16,000 ng/g) (Kieley et al. 1988) or smelting operations in Saudafijord, Norway (5,111–225,163 ng/g) (Bjorseth et al. 1979).

The wide range in ΣPAH_{24} concentrations suggests that sites with elevated concentrations may be in close proximity to recent or ongoing sources. Table 4 includes values for overall geometric means (GM) and GM +85% CL from the 1990 NS&T Mussel

TABLE 4.

Tissue organic concentrations (ng/g dry weight, mean ± SD) for Gulfwatch mussels in 1998.

Station	PAH	PCB	Chlorinated Pesticides
MASN	13.0 ± 2.0	27.2 ± 7.1	29 ± 3.0
MAIH	3330 ± 223	740 ± 39	133 ± 14.5
MAPR	553 ± 33	131 ± 7.8	60 ± 11.8
NHDP	231 ± 29	32 ± 8.5	16.1 ± 2.6
NHGP	164 ± 12.6	25.5 ± 1.5	14.1 ± 1.8
NHLH	78 ± 11.6	12.4 ± 1.8	102 ± 0.83
NHNM	645 ± 55	65 ± 9.1	67 ± 9.7
NHSS	187 ± 47	30 ± 5.4	14.6 ± 1.9
MECC	199 ± 25	42 ± 7.4	15.4 ± 2.3
MEKN	58 ± 19.6	16.7 ± 3.8	5.2 ± 0.49
MEDM	ND	3.7 ± 0.34	4.6 ± 0.50
MEBB	1120 ± 58	44 ± 5.5	61 ± 2.2
NBNR	106 ± 11.3	1.6 ± 0.18	7.0 ± 0.97
NBCH	24 ± 13.1	ND	6.5 ± 2.1
NBLN	13.8 ± 3.0	6.6 ± 0.50	5.3 ± 1.6
NBLB	16.2 ± 4.8	6.7 ± 1.3	5.8 ± 2.5
NBTC	164 ± 12.4	33 ± 5.2	13.8 ± 1.0
NBCG	229 ± 7.7	38 ± 2.3	37 ± 1.6
NSBC	133 ± 17.8	ND	5.1 ± 1.6
NSCW	138 ± 160	ND	2.5 ± 0.42
NSDI	106 ± 13.9	3.5 ± 0.43	6.0 ± 1.2
Geometric mean	127	23.5	13.6
LCL to UCL	40 to 401	8.8 to 62.6	5.9 to 31.6
NS&T Mussel Watch 1990 data (O'Connor & Beliaeff 1995)			
Geometric mean	270	110	36*
GM + 85% CI	1020	470	120*

The geometric mean and 85% confidence levels (CL) for all mussels are given below. $n = 4$ replicates per site.
ND = Not Detected.
* Value for DDT only.

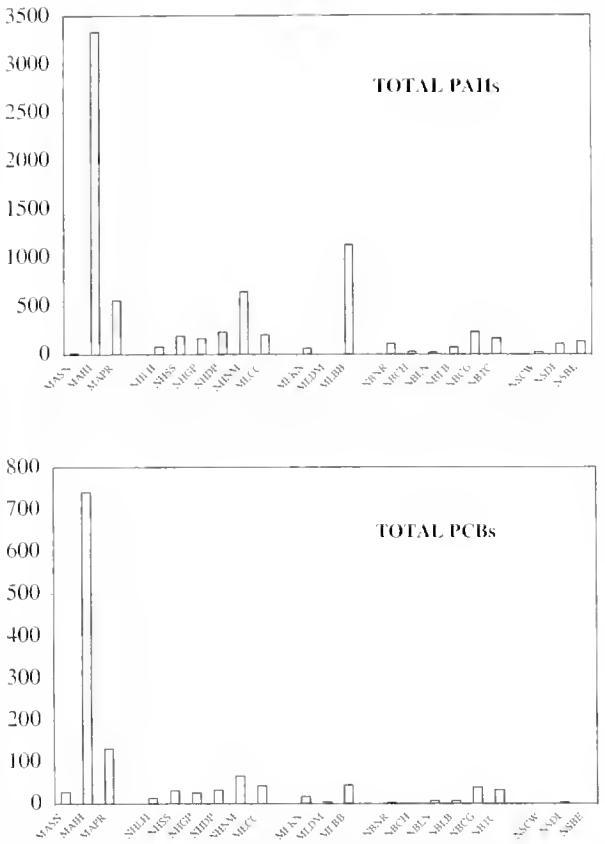


Figure 4. Distribution of total PAH and PCB tissue concentrations (arithmetic mean, µg/g dry weight) in mussels at all Gulfwatch stations in 1998.

Watch data (O'Connor & Beliaeff 1995) for comparison. Two sites, MEBB and MAIH, exceeded the NS&T GM +85% CL for PAH (1,020 ng/g DW), and two other sites, MAPR and NHNM, were at concentrations over half of this value. MAIH, and to a lesser degree MAPR, has been subject to high levels of all types of contamination. Relatively uncontaminated mussels deployed in 1995 had ~1,570 ng PAH/g DW after 60 days in cages at MAIH (Chase et al. 1996). MEBB (Boothbay Harbor, ME) had not been sampled since 1991 when organic analyses were not conducted. However, analysis of tissue samples showed mussels from MEBB to contain elevated levels of trace metals, especially Pb and Zn (Jones et al. 1998). Thus, it is a site that is apparently subject to a variety of contaminants, possibly because it is downstream from the Kennebec-Androscoggin watershed. The possible source of the PAHs at NHNM is not known. In contrast, mussels at NHDP, which was impacted by the 1996 *Provence* oil spill at the Schiller Station oil terminal, and at NHSS, which is in close proximity to the oil terminal, had much lower ΣPAH_{24} concentrations than at NHNM in 1998. Examination of the individual PAHs detected at NHNM reveals a marked dominance of higher molecular weight and nonalkylated PAHs (Chase et al. 2001). This pattern was consistent for all 1998 New Hampshire sites and suggests that the PAHs may be from pyrogenic sources, as opposed to recent petroleum sources. The pattern also strongly suggests that the sources may be historical, or reflect past exposure. Lower molecular weight PAHs degrade faster (Shiaris 1989) and are more mobile in the environment, and bivalves tend to metabolize and excrete higher molecular weight PAHs at slower rates (Widdows & Donkin 1992). Sediments from sites in North Mill Pond, especially upstream sites, had ΣPAH_{17} concentrations ranging from <690 to 23,600 ng/g DW (ANMP 1998). It is possible that PAH-contaminated sediments from upstream sources could be taken up and accumulated by mussels at the downstream NHNM site, especially during high flow or storm events at low tide.

The concentrations of ΣPCB_{24} ranged as widely as the ΣPAH_{24} concentrations. The mean of ΣPCB_{24} ranged from ND at NBCH, NSBC, and NSCW to 740 ng/g DW at MAIH (Table 4). Table 4 also shows the overall GM +85% CL of ΣPCB_{24} concentrations for all 1998 Gulfwatch sites. The same pattern of elevated concentrations in the southwest compared to the northeast sites can be seen. Two Massachusetts sites had the highest concentrations, MAIH (741 ng/g DW) and MAPR (131 ng/g DW). As described previously, MAIH is a site in Boston's Inner Harbor and has been subject to high levels of all types of contamination. Relatively uncontaminated (~37 ng ΣPCB_{24}/g DW) mussels deployed in 1995 had ~361 ng PCB/g DW after 60 days in cages at MAIH (Chase et al. 1996). In 1995, ΣPCB_{24} concentrations for MAPR, a site north of Boston Harbor, were the highest (131 ng/g DW) of any Gulf of Maine mussels sampled (Chase et al. 1996). Most of the New Hampshire sites exhibited relatively uniform and somewhat elevated concentrations relative to the Gulfwide geometric mean. The ΣPCB_{24} concentration at NHNM was the third highest of the 1998 sites at 65 ng/g DW. As with PAHs, the source of the PCBs in North Mill Pond is not known. Analysis of sediments from North Mill Pond conducted on samples collected in 1997 showed no detectable PCBs (ANMP 1998), although detection limits (>2,400 ng/g DW for seven Aroclors) were relatively high for that study. Sites in Portsmouth Harbor have had relatively high sediment PCB concentrations compared to other areas in the Gulf of Maine, except for Boston Harbor (ten Brink et al. 1997). The ΣPCB_{24} concentration in mussels at MEBB (44 ng/g DW) was also elevated compared to other 1998 sites, as was the case for other contaminants already mentioned.

It is instructive to look at data from New Hampshire as an example of how a regional database can be useful for interpreting local study results. Organic contaminant concentrations at NHNM are higher than at any other New Hampshire site (Table 4, Fig. 5), and this is a previously undocumented concern. Local community groups have focused on restoring this site, and it has been suspected to be a relatively contaminated site (ANMP 1998). However, in the context of the overall 1998 Gulfwatch database, NHNM has much lower ΣPAH_{24} and ΣPCB_{24} concentrations than at MAIH and either MAPR (ΣPCB_{24}) or MEBB (ΣPAH_{24}). Another characteristic of the New Hampshire data is that concentrations of both ΣPAH_{24} and ΣPCB_{24} are uniformly elevated compared to Gulfwatch sites north and east of New Hampshire, suggesting that there may be common sources for these relatively localized sites. Thus, interpretation of the New Hampshire data benefits greatly from comparison with the overall Gulfwatch database. Coordination of sampling and use of common laboratories for tissue analyses is a critical aspect of both programs that allows for added merit within the comparisons.

Another use of the Gulfwatch program in New Hampshire has been to document the effects of an oil spill on tissue PAH concentrations. On July 1, 1996, there was an oil spill from the vessel *Provence* into the Piscataqua River. Approximately 1,000 gallons of #6 fuel oil was dispersed with water currents into nearby areas

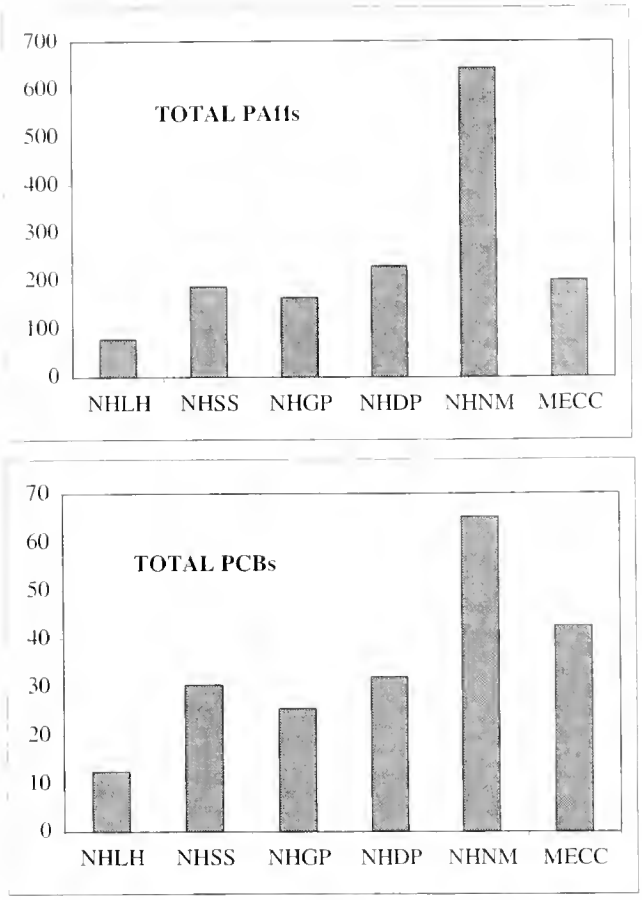


Figure 5. Distribution of total PAH and PCB tissue concentrations (arithmetic mean, µg/g dry weight) in mussels at New Hampshire Gulfwatch stations in 1998.

of the Great Bay Estuary. Fuel oils are known to contain a variety of PAHs, especially 2- to 4-ring PAHs, although hundreds of organic compounds, including larger PAHs, are present in all crude oils (Kennish 1997). The Gulfwatch station at Dover Point (NHDP), located at the confluence of the Piscataqua River and Little Bay approximately 2.5 miles upstream of the source of the oil spill, was sampled in 1994. Sampling was again conducted in July and October 1996 to determine if contaminants from the spill were taken up by mussels and to find the degree of elimination of the contaminants over time. The 1994 data serve as useful background information for assessing the degree of residual exposure of the 1996, 1997, and 1998 mussel tissue samples to the oil spill contaminants. ΣPAH_{24} concentrations were determined in mussel tissue samples collected in 1994, in 1996 on July 16 and October 1, 16 days and 3 mo, respectively, after the spill, and in 1997 and 1998 (Fig. 6). ΣPAH_{24} concentrations in the 1996/16-day samples were much higher than in 1994, as well as in comparison to other sites in New Hampshire. Elevated levels of PAHs were also observed in oysters (*Crassostrea virginica*) collected at another site impacted by the spill (Chase et al. 1997). The average ΣPAH_{24} concentrations were 639, 298, 266, 238, and 187 ng/g DW for the 16-day, 3 mo, 1997, 1998, and 1994 samples, respectively. Thus, the total PAH concentration has decreased greatly from 1996, less drastically from 1997 to 1998, and levels have almost returned to prespill (1994) levels.

Temporal Variation in Contaminant Concentrations

Sampling has occurred in up to five previous years at the five "benchmark" Gulfwatch stations to provide data for temporal trend analysis (Jones et al. 1998). Data for MECC, a benchmark station sampled since 1993, are presented here as one illustration of temporal trends in the Gulf of Maine (Table 5). There were few obvious trends in concentrations of trace metals and organic contaminants. Trends at the other four benchmark sites also showed variable results, with most discernable trends being decreases in contaminant concentrations. There has been significant variation for between-year comparisons for some contaminants (Chase et al. 2001).

Acceptable Levels and Standards of Mussel Contamination

Despite the wealth of information on the effects of toxic contaminants on a variety of species, limited information is available on observed human health effects resulting from the consumption of chemically contaminated shellfish. While documented epidemiological effects may be limited, the results of laboratory assays and isolated occurrences of acute human poisonings are responsible for focusing attention on human health impacts from eating chemically contaminated marine fish and shellfish. For example, in New Hampshire there are currently human consumption advisories for Hg and PCBs (NHDES 1998, NHEP 2001). The advisory for Hg is based on elevated Hg levels in inland lakes and rivers and is for all freshwater fish. For marine waters, there is a consumption advisory for both lobsters and bluefish based on elevated levels of PCBs. The PCB advisories for bluefish and lobsters are based on studies done in 1987 and 1991, respectively.

Published tolerance or action levels for PAHs in commercial marine species are not available in Canada or in the United States. Closure of commercial fisheries as a result of high contamination levels has been dealt with on a case-by-case basis in marine areas where PAH contamination may be a human health concern. In general, most concentrations reported in the literature are on a wet weight basis in contrast to Gulfwatch dry weight values. To facilitate general comparisons with Gulfwatch values, an average moisture content of 85% has been applied to wet weight health values to derive dry weight equivalents. All Gulfwatch organic concentrations are within acceptable concentrations for those compounds that have established FDA Action Limits in fish and shellfish. PCB concentrations found in Gulfwatch mussels (Table 4), including concentrations measured in MAIH mussels (0.74 ± 0.003 µg/g DW) are less than the action level of 13 µg/g dry weight (USFDA 1990, CSSP 1992). The action level for the pesticides dieldrin, aldrin, chlordane, heptachlor, and heptachlor epoxide is 2.0 µg/g dry weight (USFDA 1990). Only dieldrin and chlordane were detected in the 1998 mussel survey, but at concentrations barely above detection limits, which are orders of magnitude below the action levels. The total DDT concentrations found are several orders of magnitude below the action level of 33 µg/g dry weight (USFDA 1990, CSSP 1992). Canadian limits for agricultural chemicals exclusive of DDT are 0.67 µg/g DW.

As presented in Table 6, admissible levels of methyl mercury, expressed as µg Hg, are less than 6.7 µg/g dry weight, or 1 µg/g wet weight in the United States (USFDA 1990), and less than 3.3 µg/g dry weight, or 0.5 µg/g wet weight in Canada (CSSP 1992). The highest concentration of mercury found in the 1998 Gulfwatch study was 1.20 µg/g DW, in one replicate sample from the Schiller

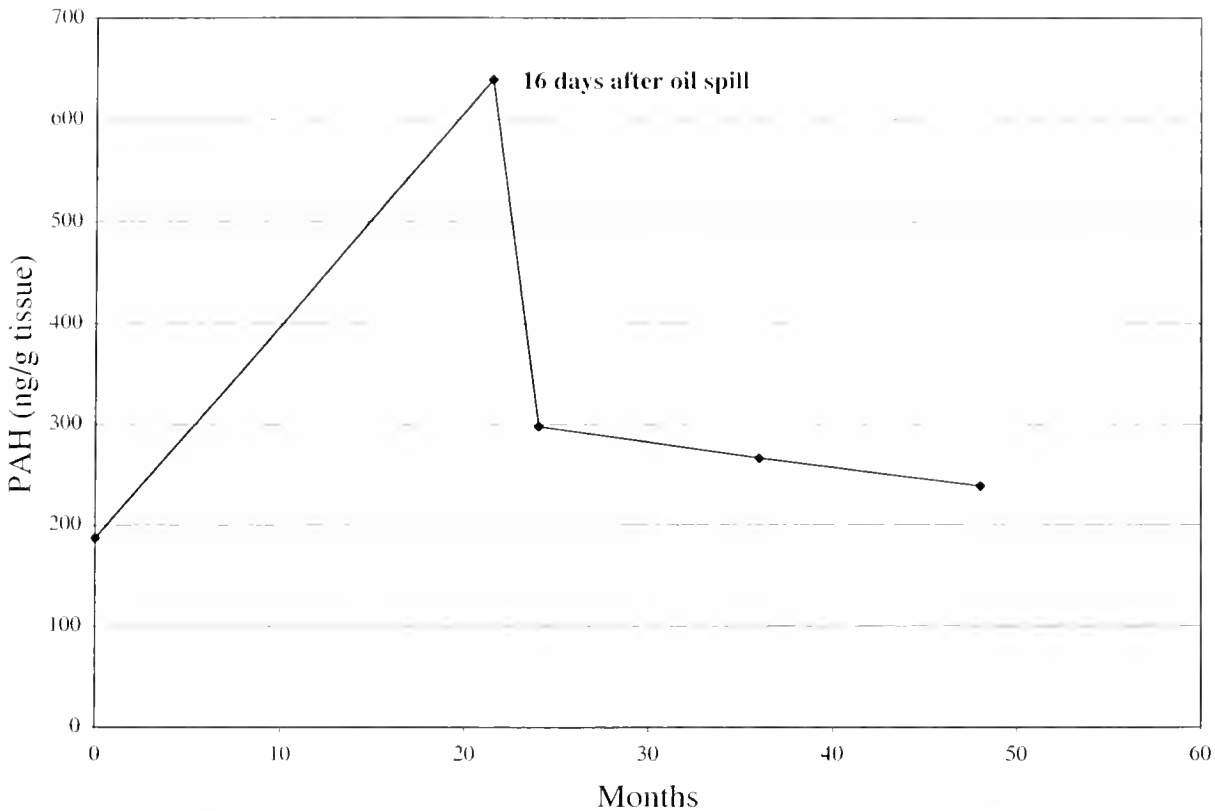


Figure 6. Long-term effects of a July 1996 oil spill on PAH contamination in mussels at Dover Point, NH. First PAH concentration sample collected in October 1994.

Station, New Hampshire, which is well below both federal action concentrations.

A series of FDA "Guidance Documents" (USFDA 1993) for cadmium, chromium, lead, and nickel was released in the United States to complement the FDA Mercury Action Level. These "alert" levels are guidelines and by themselves do not warrant the issuance of health advisories. In Table 6, guidance concentrations are reported on both wet weight and dry weight bases and are compared to the highest observed concentration in any single replicate analyzed in the 1998 Gulfwatch Project. All nickel, chromium, and cadmium concentrations in 1998 Gulfwatch mussels were well below the guideline values. However, Pb concentrations were above the FDA guideline alert level of 11.5 μg/g DW at MAIH and MEBB, and are thus of regional and local concern. The highest observed concentrations from the 1998 Gulfwatch data for other trace metals for which there is no guideline or action limit are included in Table 6. This highlights hot spots of localized elevated contamination as well as sites where elevated levels may also be

TABLE 5.

Long-term tissue contaminant concentrations (arithmetic mean ± SD) for a Gulfwatch baseline station: Clark Cove, ME (MECC).

Contaminant	1993	1994	1995	1996	1997	1998
Metals (μg/g dry weight)						
Ag	0.10 ± 0.05	0.07 ± 0.03	0.06 ± 0.03	0.05 ± 0.02	0.04 ± 0.02	0.03 ± 0.01
Cd	2.38 ± 0.27	1.33 ± 1.50	1.37 ± 0.87	1.26 ± 0.27	0.94 ± 0.50	0.74 ± 0.44
Cr	3.31 ± 1.28	2.29 ± 1.44	2.08 ± 0.09	1.69 ± 0.62	1.33 ± 0.67	1.08 ± 0.52
Cu	7.51 ± 0.87	4.19 ± 4.70	4.31 ± 2.72	3.98 ± 0.87	2.97 ± 1.56	2.34 ± 1.40
Pb	5.35 ± 2.18	3.77 ± 2.24	3.39 ± 1.50	2.72 ± 1.04	2.16 ± 1.08	1.75 ± 0.83
Hg	0.74 ± 0.06	0.40 ± 0.48	0.42 ± 0.28	0.40 ± 0.08	0.30 ± 0.15	0.23 ± 0.14
Ni	2.60 ± 0.2	1.40 ± 1.7	1.47 ± 0.99	1.39 ± 0.29	1.04 ± 0.54	0.81 ± 0.49
Zn	126 ± 17	71 ± 77	73 ± 45	66 ± 15	50 ± 26	39 ± 23
Al	187 ± 81	134 ± 75	119 ± 52	95 ± 38	76 ± 38	62 ± 29
Fe	535 ± 138	336 ± 281	322 ± 164	276 ± 78	210 ± 110	169 ± 91
Organics (ng/g dry weight)						
PAH	154 ± 47	137 ± 10	158 ± 39	203 ± 22	147 ± 19	200 ± 26
PCB	70 ± 11	67 ± 5	35 ± 10	38 ± 2	37 ± 8	42 ± 8
Pesticides	11.1 ± 5.3	12.5 ± 1.3	13.8 ± 1.0	7.2 ± 1.5	15 ± 5	16 ± 2

TABLE 6.

Comparisons of U.S. Food and Drug Administration federal guideline and action concentrations for trace metals with the Gulfwatch results, and the highest observed 1998 Gulfwatch concentrations.

Metal	Wet Weight	Dry Weight	Highest Observed 1998 Gulfwatch Value (Dry Weight)	Location
Guideline concentration				
Cd	3.7 μg/g	25 μg/g	3.1 μg/g	Cornwallis, NS
Cr*	13 μg/g	87 μg/g	19.7 μg/g	Tin Can Beach, NB
			4.2 μg/g	Little Harbor, NH
Pb	1.7 μg/g	11.5 μg/g	37 μg/g†	Boston Inner Harbor, MA
			18 μg/g†	Boothbay Harbor, ME
Ni*	80 μg/g	533 μg/g	9.4 μg/g	Tin Can Beach, NB
			2.1 μg/g	Cornwallis, NS
Federal action concentration				
Hg (US)	1.0 μg/g	6.7 μg/g	1.2 μg/g	Schiller Station, NH
Hg (CA)	0.5 μg/g	3.3 μg/g		
No federal guideline or action concentration				
Highest observed 1998 Gulfwatch value				
Ag*		2.0/1.3		Coast Guard wharf, NB/Sandwich, MA
Al*		5640/440		Tin Can Beach, NB/Cornwallis, NS
Cu*		43/26		Coast Guard wharf, NB/Boston, Inner Harbor, MA
Fe*		2950/610		Tin Can Beach, NB/Boston Inner Harbor, MA
Zn		310		Boston Inner Harbor, MA

* Two values for some metals are presented because the highest values are all from New Brunswick sites where tissue samples tended to contain excessive inorganic sediment, as indicated by elevated Al and Fe concentrations.

† Gulfwatch highest values exceed U.S. FDA Guideline concentration. Four replicate sample concentrations ranged from 30–37 μg/g DW at MAIH and 13–18 μg/g DW at MEBB.

associated with excessive sediment in tissue samples (New Brunswick sites). Many of the areas with elevated concentrations are currently under more detailed investigations by other agencies.

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DISTRIBUTION PATTERNS OF POLYCHLORINATED BIPHENYL CONGENERS IN MARINE SEDIMENTS AND WILD MUSSELS FROM GALICIA COAST (NORTHWESTERN SPAIN)

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ABSTRACT To know distribution patterns of polychlorinated biphenyl congeners in the marine environment from Galicia, polychlorinated biphenyls (PCBs) no. 31, 28, 52, 101, 118, 153, 105, 138, 156, and 180 were isolated by Soxhlet extraction in wild mussels and surface marine sediments; the quantification was performed using gas chromatography-mass spectrometry (GC-MS) and gas chromatography-electron capture detection (GC-ECD). PCBs 101, 138, 153, and 180 were the most abundant congeners in all samples analyzed. Distribution of PCB congeners was shifted in favor of higher chlorinated compounds. By means of multivariate techniques of data exploration, such as hierarchical cluster analysis and principal components analysis, mussel samples from contaminated (Ferrol and A Coruña zones) areas were clearly separated from uncontaminated (all the other samples) areas. Sediment samples were more heterogeneous. If tri- and tetrachlorinated biphenyls were considered when carrying out the multivariate analysis, Vilaboa samples formed a group differentiated from the other samples.

KEY WORDS: polychlorinated biphenyls (PCBs), wild mussels, marine sediments, principal components analysis (PCA), hierarchical cluster analysis (HCA)

INTRODUCTION

The increase of human activity and industrial development in Galicia is intensely disturbing the natural marine environment. Its progressive deterioration is mainly caused by contributions of various different industrial discharges that reach the coast without previous treatment or purification. Taking into account the high importance of shellfishing in Galicia, especially bivalve mollusks (the aquaculture of mussels is one of the main resources), it is necessary to carry out a baseline analysis of micropollutants in the estuarine bays ("Rías") from Galicia.

Polychlorinated biphenyls (PCBs) are considered to be thermally, chemically, and physically stable micropollutants; this stability has been responsible for PCB marine environmental contamination problems (Erickson 1997). PCB compounds are volatile, lipophilic, and persistent. They tend to bioaccumulate and bioconcentrate in fatty tissues of biota and to associate with benthic and suspended sediments (Berger & Brevik 1996, Beyer et al. 1996). Mussels, with low PCB metabolism, are a good coastal indicator for micropollutants in the water column and sediments.

Various instrumental techniques are available for the extraction and determination of PCBs in environmental samples. Techniques for the extraction of PCBs include Soxhlet extraction (Piñeiro et al. 1996), microwave-assisted extraction (MAE) (Carro et al. 2000, Carro et al. 1999b), Supercritical fluid extraction (SFE) (Hawthorne et al. 1993, Soren & Berit 1994), solid phase microextraction (SPME) (Llompart et al. 1999), solid phase extraction (SPE), accelerated solvent extraction (ASE) (Bandh et al. 1998) and others; techniques for the determination include gas chromatography-electron capture detection (GC-ECD) (Chen & Ling 1992), gas chromatography-mass spectrometry (GC-MS) (Pavoni et al. 1991, Leonards et al. 1996, Shin & Oh-Shin 1999), gas chromatography-atomic emission detection (GC-AED), gas chromatography-Fourier transform infrared (GC-FTIR) (Hembree et al. 1993), and others. Previous works on distribution of chlorinated biphenyls in mollusks from some Galicia estuarine bays were developed using different extraction methods (Fumega et al. 1984, Franco et al. 1984, Fernández Muiño et al. 1991, Alvárez Piñeiro et al. 1994, Alvárez Piñeiro et al. 1995).

The objective of this study was to describe the distribution of PCBs in wild mussels (*Mytilus galloprovincialis*) and in surface marine sediments collected from 19 sites in the Galicia coast. Ten PCB compounds (IUPAC No. 31, 28, 52, 101, 118, 153, 105, 138, 156, and 180) recommended by the International Council for Exploration of the Seas (ICES) (Duinker et al. 1988) have been isolated using Soxhlet extraction and analyzed by GC-ECD and GC-MS. Multivariate techniques of data exploration as hierarchical cluster analysis (HCA) and principal component analysis (PCA) have been applied to sediments and mussels data.

MATERIALS AND METHODS

Materials and Apparatus

Dichloromethane, n-pentane, and isooctane for organic trace analysis were purchased from Merck (Darmstadt, Germany); aluminum oxide, silica gel, and anhydrous sodium sulfate were also purchased from Merck. Copper turnings were supplied by Aldrich (Steinheim, Germany). Analytical reagent grade PCB individual congener standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany).

Wild mussels (*Mytilus galloprovincialis*) and surface sediments were collected from 19 coastal subzones in the Estuarine Bays ("Rías") from Galicia (December 1999): Ribadeo (Ría de Ribadeo), Foz (Ría de Foz), and O Vicedo (Ría do Barqueiro) from Lugo zone (samples labeled as 1); As Pías Montón, As Pías Puntal, Barallobre, and Mugardos (Ría de Ferrol), Pontedeume (Ría de Ares), and Miño (Ría de Betanzos) from Ferrol zone (samples labeled as 2); Santa Cruz and Pasaxe (Ría de A Coruña) from A Coruña zone (sample labeled as 3); Anllóns (Ría de Corme-Laxe), Camariñas (Ría de Camariñas), and Corcubión (Ría de Corcubión) from Costa da Morte zone (samples labeled as 4); Raxó and Lourizán (Ría de Pontevedra), Vilaboa, Arcade, and Baiona (Ría de Vigo) from Pontevedra zone (samples labeled as 5) (see Fig. 1).

Two certified reference materials, HS-2 (Canadian marine sediment) supplied by National Research Council of Canada and IAEA 142 (mussel tissue homogenate) supplied by International Atomic

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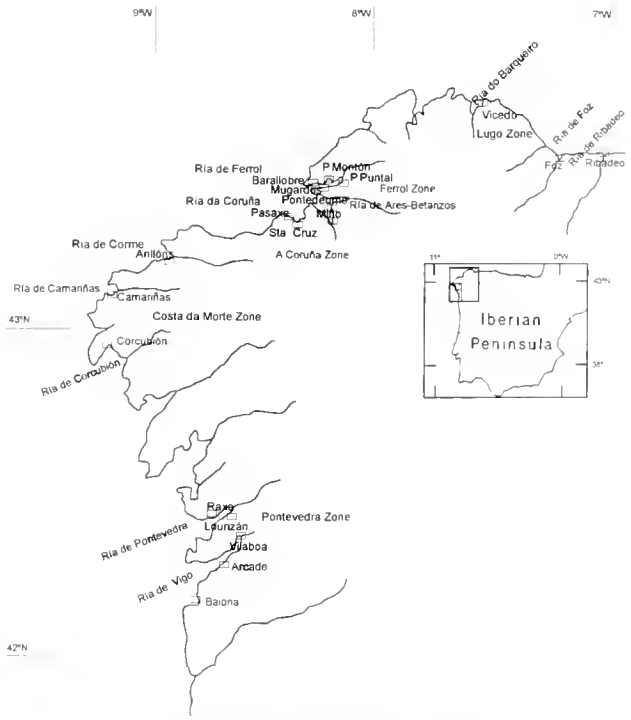

Figure 1. Map with sampling points.

Energy Agency (Vienna, Austria), were used for quality control. Standard stock solutions were prepared by weighing an appropriate amount of each standard and diluting to 5 mL with isooctane. Working solutions were made by appropriate dilution of the stock solution. All solutions were stored at 4 C. For quantitative gas chromatographic determinations, calibration was carried out at four concentration levels for each congener spanning the range of 4–100 μL and using CB 155 (1 mg/L) as an internal standard.

The concentrated extracts were analyzed by gas chromatography using a Perkin-Elmer Autosystem gas chromatograph equipped with an electron capture detector. A TRB-5 (Teknokroma, Spain) 5% diphenyldimethyl siloxane capillary column (60 m × 0.20 mm i.d. × 0.4 μm phase thickness) was used. The chromatographic conditions were as follows: the column temperature program was 90°C (3 min) to 215°C (40 min) at 30°C/min and 275°C (30 min) at 5°C/min; the injector temperature (splitless mode, 1.8 min) was 270°C; the electron capture detector temperature was 365°C; carrier gas was hydrogen supplied by Air Liquid (Spain). The identification of extracted compounds was performed on a Varian Saturn 2000 gas chromatograph-ion trap detector mass spectrometer. The chromatographic conditions and capillary column were identical to those described above; carrier gas was helium supplied by Air Liquid (Spain). The mass conditions were: the trap temperature 170°C; the scan range 100–400 m/z; the multiplier delay 5 min; the emission current 10 μA; the multiplier voltage 1,450 V; the maximum ionization time 25,000 μsec; and the manifold temperature 120°C.

Data numerical analysis was carried out by means of the statistical package (SPSS Inc.; 1989–1999 V.9.0).

Sample Preparation

Thirty individual mussels of similar sizes (40–80 mm) were used for each analysis. Homogenates of the mussel flesh were frozen (-30°C), freeze-dried and Soxhlet extracted (5 g of sample; 150 mL of dichloromethane-pentane, 1–1; 8 h) by duplicate, and the extract was concentrated under vacuum evaporation to approximately 1 mL. An aliquot of the extract was used to determine gravimetrically the lipid content. Lipids were removed from an appropriate portion of the concentrate by chromatography over alumina (6% deactivated) and the CB fraction was purified by chromatography on silica (1% deactivated). CB 155 was added as an internal standard prior to analysis by gas chromatography.

Sediments were collected by hand using a metal scoop at 0–30 cm depth and immediately frozen (−30°C). The sediments were freeze-dried and sieved to obtain a particle size below 63 μm. Activated copper turnings were used to remove elemental sulfur during Soxhlet extraction (5 g of sample; 150 mL of dichloromethane-pentane, 1–1; 8 h) in duplicate. The sediment extracts were concentrated under vacuum evaporation to 1 mL and were treated in the same manner as the mussel samples.

RESULTS AND DISCUSSION

Validation of Analytical Procedure of PCB Compounds

Calibration curves were obtained at four concentration levels using adequately diluted standards. Each concentration level was injected in duplicate, and peak heights were fitted by linear regression. The correlation coefficient was 0.999 for all the target compounds.

Method reproducibility experiments were carried out in five replicate mussel and sediment samples, providing a mean relative standard deviation of 8.8% and 12.71%, respectively; the mean recoveries of PCBs were 92.09% and 101.31% from mussel and sediment samples, respectively.

Individual Congener Concentrations: Univariate Analysis

Concentrations of individual congeners of PCBs (IUPAC No. 31, 28, 52, 101, 118, 153, 105, 138, 156, and 180) expressed as the average of two analytical replicates in ng/g dry weight (dw) from mussels and marine sediments and lipid content of mussels in percentage of studied subzones are summarized in Tables 1 and 2.

The contents of PCBs in wild mussels and marine sediments varied from 0.06 ng/g to 115 ng/g dw and from 0.01 to 19.4 ng/g dw, respectively. In both mussels and marine sediments, congeners making the largest individual contributions to total congener concentration (sum of 10 congeners) were the penta-, hexa-, and heptachlorobiphenyls (PCBs 101, 138, 153, and 180); these compounds are less volatile, more resistant to metabolic and microbial degradation, adsorb readily to sediments, and are more soluble in lipids than lower chlorinated congeners (Shiu & Mackay 1986). The low concentrations of tri- and tetrachlorobiphenyls (PCBs 28, 31, and 52) in samples were due to their higher volatility, low partition coefficient between n-octanol and water (greater aqueous solubility), and higher tendency to biodegradation in marine sediments (Bright et al. 1995). However, the Vilaboa subzone showed very high levels of lower chlorinated compounds, mainly in the sediment sample; these results will be discussed below.

In general, concentrations of PCBs in mussels coming from the same zone were very homogenous; levels of PCBs in mussels collected from Ferrol and A Coruña zones (labeled as 2 and 3, respectively), which possessed the greatest lipid contents, were higher than those found in the other zones; it is logical because these areas present the largest degree of urbanization and indus-

TABLE 1.

Mean of concentration of PCBs (ng/gdw) and lipid content (%) in mussels from Galicia coast.

Zone	Subzone	Cb31	Cb28	Cb52	Cb101	Cb118	Cb153	Cb105	Cb138	Cb156	Cb180	Lipid Content
1	Ribadeo	0.18	0.19	0.8	2.47	1.78	12.94	0.5	6.63	0.52	2.06	5.85
1	Foz	0.15	0.38	0.24	1.32	1.12	7.33	0.28	3.98	0.28	1.43	5.72
1	O Vicedo	0.09	0.06	0.22	3.79	2.01	47.62	—	24.35	2.12	7.93	4.3
2	Pias Monton	0.79	1.77	3.57	19.47	17.36	87.67	6.47	50.38	3	10.9	8.58
2	Pias Puntal	0.7	1.44	3.27	21.15	23.53	106.21	4.73	64.12	3.95	13.91	8.65
2	Barallobre	0.51	0.88	4.07	22.16	19.79	87.38	3.21	47.28	3.11	7.49	7.55
2	Mugardos	0.61	1.44	3.13	20.25	23.08	115.52	4.36	64.16	3.47	12.35	10.95
2	Pontedeume	0.2	0.28	0.82	4.29	3.54	26.38	0.79	14.76	0.81	2.29	9.4
2	Miño	0.21	0.11	0.57	3.87	4.18	33.78	0.86	19.54	0.61	3.6	6.5
3	Sta. Cruz	0.39	0.76	0.92	7.74	8.73	67.33	1.74	36.82	2.58	13.17	6.06
3	Pasaxe	0.36	0.6	1.46	10.2	7.75	79.06	1.81	51.41	3.25	14	6.99
4	Anllóns	0.46	0.59	1.04	5.15	1.26	9.08	0.63	4.73	0.85	1.08	6.34
4	Camariñas	0.19	0.29	0.43	2.36	2.45	11.36	0.62	6.67	0.4	1.48	5.27
4	Corcubión	0.71	1.15	1.86	4.89	4.99	13.29	1.17	9.57	0.57	1.43	6.4
5	Raxó	0.28	0.55	1.22	5.03	5.83	17.49	2.55	11.76	0.77	2.58	8.48
5	Lourizán	0.43	0.94	1.26	6.9	5.8	28.2	2.7	18.98	1.76	7.39	8.22
5	Vilaboa	2.26	3.5	2.6	6.49	6.06	33.85	1.23	20.03	1.7	5.48	6.83
5	Arcade	0.19	0.37	0.58	4.76	4.54	33.6	0.94	18.63	1.14	4.16	7.8
5	Baiona	0.77	0.66	0.44	2.56	3.45	8.08	0.81	5.67	0.53	0.91	6.49

trialization in Galicia. PCB concentrations in sediment samples showed a greater intrazonal heterogeneity.

To select the most significant variable to distinguish between the studied zones, data in Tables 1 and 2 were subjected to univariate analysis. A one-way analysis of variance (ANOVA) revealed that CB153 (present in enhanced concentrations in industrial mixtures) was the most significant variable for mussel and sediment samples; Figure 2 shows the box-whisker plot corresponding to this variable.

Congener Distribution Patterns: Multivariate Analysis

In view of the previous results, it was logical to use the set of variables at hand to achieve more precise distinction of the zones. Hence, the data (in the case of mussels, normalized to lipid content) in Tables 1 and 2 were subjected to PCA and HCA. Initially, both types of multivariate analysis were applied separately to data for the mussel and sediment samples.

Mussel Samples

Cluster analysis of data (concentration of higher chlorinated biphenyls, CBs 101, 118, 153, 105, 138, 156, and 180) for the mussel samples using the average linkage method led to two fairly robust clusters (Fig. 3A). The most compact cluster was formed by the samples from Ría de Ferrol (zone labeled as 2) and Ría de A Coruña (zone labeled as 3), the most contaminated zones; at higher

TABLE 2.

Mean of concentration of PCBs (ng/g dw) in sediments from Galicia coast.

Zone	Subzone	Cb31	Cb28	Cb52	Cb101	CB118	Cb153	CB105	Cb138	Cb156	Cb180
1	Ribadeo	—	—	0.04	0.2	—	1.35	—	0.57	0.05	0.61
1	Foz	—	—	0.12	—	—	0.24	—	—	—	—
1	O Vicedo	—	—	—	0.04	—	0.95	0.27	0.45	0.05	0.87
2	As pias Monton	0.09	0.19	0.42	1.61	0.95	5.69	0.2	3.59	0.32	7.45
2	As pias Puntal	—	—	0.04	0.16	—	0.97	—	0.39	0.01	0.35
2	Barallobre	—	—	0.83	6.38	1.35	16.22	0.17	8.12	0.8	8.35
2	Mugardos	0.02	0.19	0.26	3.95	0.79	11.4	0.14	5.49	0.52	5.07
2	Pontedeume		—	0.52	0.88	0.4	0.96	0.1	0.65	—	0.25
2	Miño	0.07	0.14	0.21	0.26	—	0.82	—	0.38	0.01	0.28
3	Sta Cruz	—	—	0.03	0.17	—	0.72	—	0.36	—	0.38
3	Pasaxe	—	—	0.13	1.38	0.25	5.8	—	3.41	0.36	6.04
4	Anllóns	—	—	0.03	—	—	1.41	—	—	—	—
4	Carmariñas	0.17	0.45	0.55	0.72	0.1	1.28	—	0.59	0.01	0.23
4	Corcubión	—	—	0.03	0.11	—	0.99	—	0.5	0.05	0.93
5	Raxo	—	—	0.01	—	—	0.6	—	0.02	—	0.11
5	Lourizán	0.03	0.05	0.19	4.75	1.05	19.4	—	12.06	1.25	13.96
5	Vilaboa	3.44	5.28	2.15	1.7	0.57	5.73	0.33	3.6	0.27	5.19
5	Arcade	—	—	0.07	0.26	—	0.73	—	0.3	—	0.02
5	Baiona	—	—	0.03	0.01	—	0.61	—	—	—	—

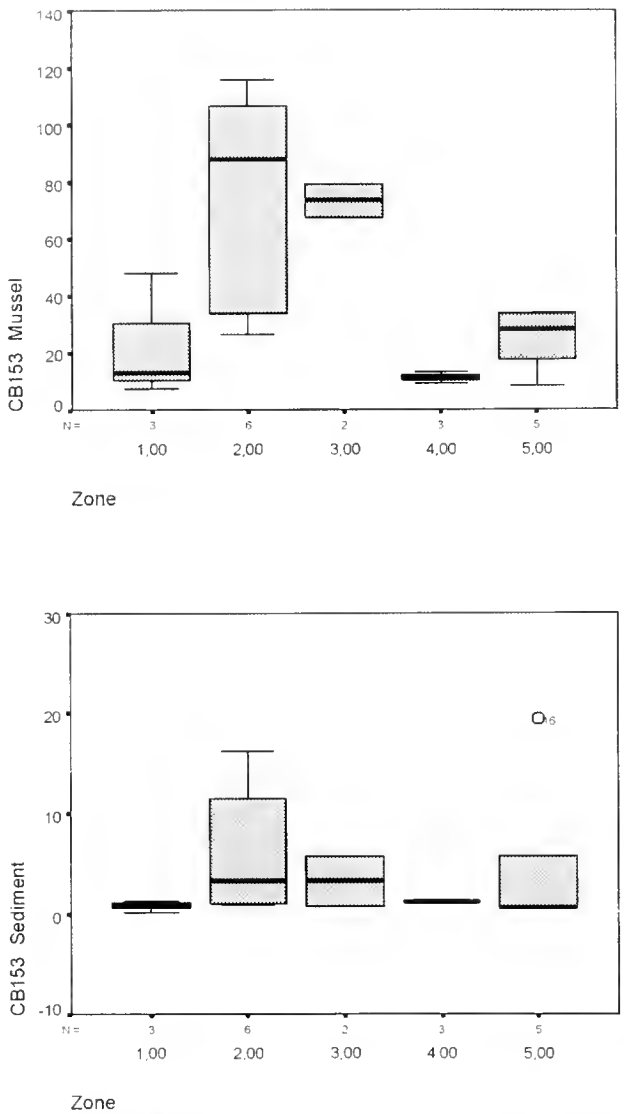

Figure 2. Box-whisker plot of CB153 for the five sampling zones (mussel and sediment samples). Data are divided into areas of equal frequency. Box: middle 50% frequency. Lower whisker: from the first quartile to the smallest data point. Upper whisker: from the third quartile to the largest data point.

similarity, these samples were separated in two new clusters that grouped mussels from Ría de Ferrol and Ría de A Coruña, respectively. The other principal cluster (less compact) was formed by samples from slightly contaminated zones. Mussel samples from Pontedeume and Miño subzones (Ría de Ares and Ría de Betanzos, respectively) belonging to Ferrol zone (labeled as 2) were placed in this cluster. If tri- and tetrachlorobiphenyls (CBs 31, 28, and 52) were used to carry out the cluster analysis, mussels from Vilaboa subzone (Pontevedra zone, labeled as 5) were clearly differentiated from all the others (Fig. 3B).

The same conclusions are extracted using PCA: retaining the first two factors, 85.93% of the initial variance was explained (PC1, 66.42%; PC2, 19.51%). Figure 4 shows the projection of the objects (sampling subzones) on the plane of the first two components. Ría de Ferrol and Ría de A Coruña samples were associated with the positive part of the first component that comprised the penta-, hexa-, and heptachlorobiphenyls. The distribution of the uncontaminated samples group was mainly influenced by the negative part of the first factor. The Vilaboa sample was associated with the second component that was constructed with the CBs 31, 28, and 52 contribution.

In mussel samples, CB 153 and the other higher polychlorinated biphenyls showed a clear differentiation between contaminated and uncontaminated zones independently of the geographical proximity. Pollution of industrial zones does not reach the nonindustrialized sites; this behavior can be mainly observed in Ferrol zone. Pontedeume and Miño subzones are slightly contaminated and are considered as unspoiled and not industrialized areas, while the other subzones (P. Puntal, P. Montón, Mugardos, and Barallobre) are highly contaminated industrial and urbanized areas. Actually, there are no studies of water flows in the Ferrol zone but the distribution of PCB compounds suggest that the water circulation of the estuarine bays (Ares, Betanzos, and Ferrol) is very slow in this area.

Lourizán and Vilaboa subzones presented high contents of PCBs. Lourizán is placed near to Pontevedra city with an important pulp paper and chlorine bleaching mill; Vilaboa is sited near to the most industrialized city in Galicia (Vigo) and placed in a very closed zone of the estuarine bay.

Sediment Samples

Cluster analysis of data (sum of congeners) for the sediment samples using the average linkage method (Fig. 5) distinguished two groups. One of the groups included the most contaminated sediments, Barallobre subzone (sample labeled as 2) and Lourizán subzone (sample labeled as 5). The other group consisted of two subgroups. The first subgroup included the slightly contaminated samples Mugardos and Montón subzones (samples labeled as 2) and Pasaxe subzone (sample labeled as 3) and Vilaboa subzone (sample labeled as 5). The second subgroup included the least contaminated samples. Analytical results for all of the sediment samples were more heterogeneous than for the mussel samples.

Sediment samples from Lourizán and Vilaboa subzones with a high content of total organic carbon (TOC) (silty muds) presented a greater concentration of PCBs in comparison with expected values of concentration from their mussel samples. This could be due to the particular conditions developed in these subzones that produce a low PCBs bioavailability from sediments to mollusks; these subzones are sited in very stagnant areas in the estuarine bay where redissolution of organic compounds from sea bottom to water is improbable. Moreover, the high content of total organic carbon in sediments causes a high accumulation of PCBs (hydrophobic contaminants) because they tend to be strongly associated with organic-rich particles. These sediments can act as a temporary pollutant trap before accumulation in biota. In general, the bioavailability and toxicity of sediment-associated contaminants decrease with increasing TOC content (Lake et al. 1990). In further research, the TOC values of sediments (with different structures and compositions) will be considered to attain a good geographical comparison and a correct data interpretation in contamination studies. The sediment TOC and the lipid content of the organisms are key factors in the partitioning of organic contaminants.

Mussels from Puntal (sample labeled as 2) and Sta Cristina (sample labeled as 3) subzones had a higher concentration of PCBs than their corresponding sediment samples. This could be due to the low depth of sampled sediments (0–30 cm). In these subzones, the surface sediment, placed in a tidewater zone, is a "clean" sandy

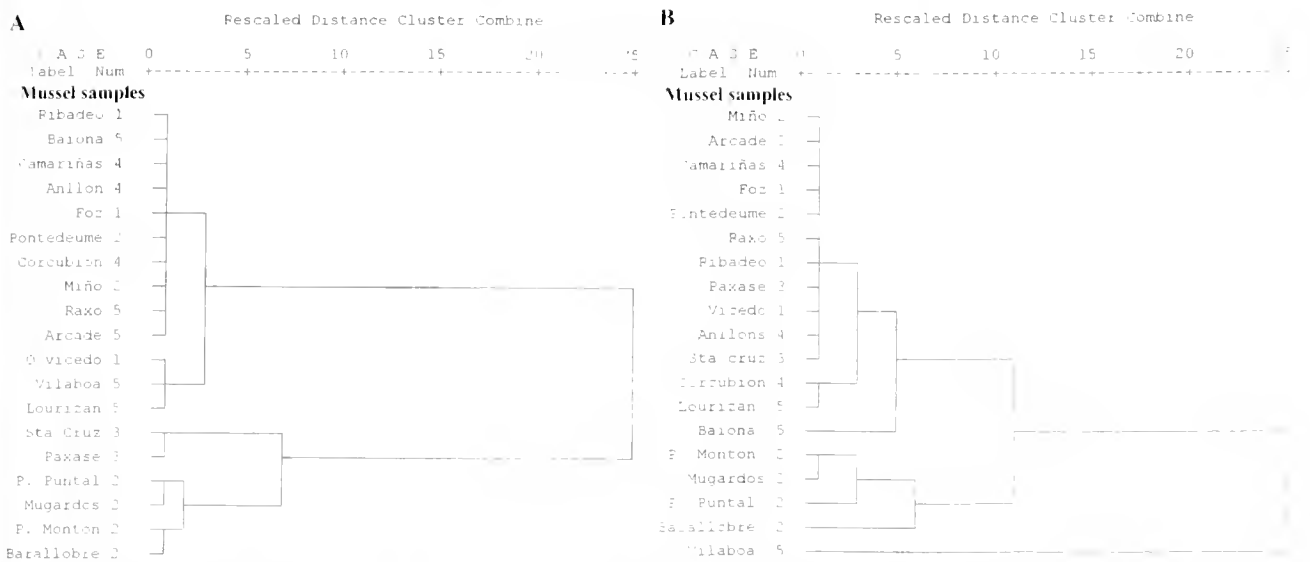

Figure 3. (A) Dendrogram showing the results of a hierarchical cluster analysis on higher chlorinated biphenyl distributions in Galicia mussels. (B) Dendrogram showing the results of a hierarchical cluster analysis on lower chlorinated biphenyl distributions in Galicia mussels.

deposit with very low levels of TOC and organic pollutants. Sometimes, accumulation of contaminants in several species (including mussels) does not depend on pollutant concentration in sediments but it can be strongly affected by a selective uptake of contaminated food. In these zones, the local (urban) point discharges are frequent.

If only tri- and tetrachlorobiphenyls CBs 28, 31, and 52 were considered when carrying out the cluster analysis, the sample from Vilaboa was clearly differentiated from all the others; the behavior was similar to the mussel sample.

Two principal components were found to account for about 91.25% of the initial variance. The most contaminated group is associated with the first component (constructed with penta-, hexa-, and heptachlorinated biphenyls). The Vilaboa sample is related to the positive part of the first and second components (the latter is constructed with tri- and tetrachlorinated biphenyls).

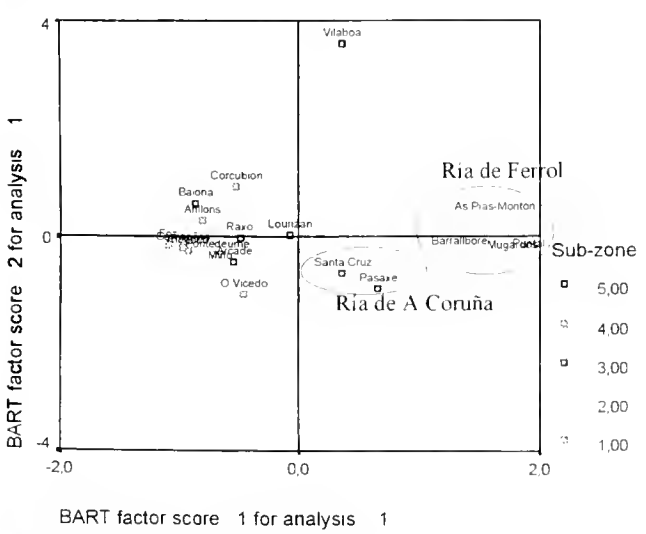

Figure 4. Distribution of mussels in the plane of the first and second component.

Correlation between Mussels and Sediments from the Same Zone

Due to the cycling of micropollutants in marine inlets, mussels and marine sediments must be significantly correlated in a unique manner that should predict the general distribution patterns of PCBs. In this way, the possible sources of organic micropollutants can be detected and actions initiated.

To confirm this hypothesis, the data sets in Tables 1 and 2 were subjected to multivariate analysis. In general, cluster analysis in Figure 6A shows separation between sediments and mussels, but there are some slightly contaminated mussels that were placed in the sediment cluster and some highly contaminated sediments (Lourizán subzone) that were placed in the mussel cluster (Baiona, Camariñas, Anllón subzones). Mussels of Ría de Ferrol were clearly separated from all samples.

If only tri- and tetrachlorinated biphenyls are considered, Vila-

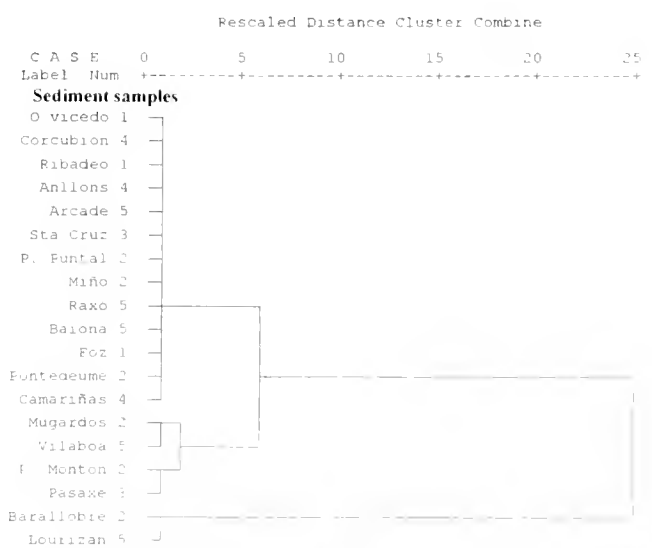

Figure 5. Dendrogram of Galicia sediments. Sum of congeners was considered to carry out cluster analysis.

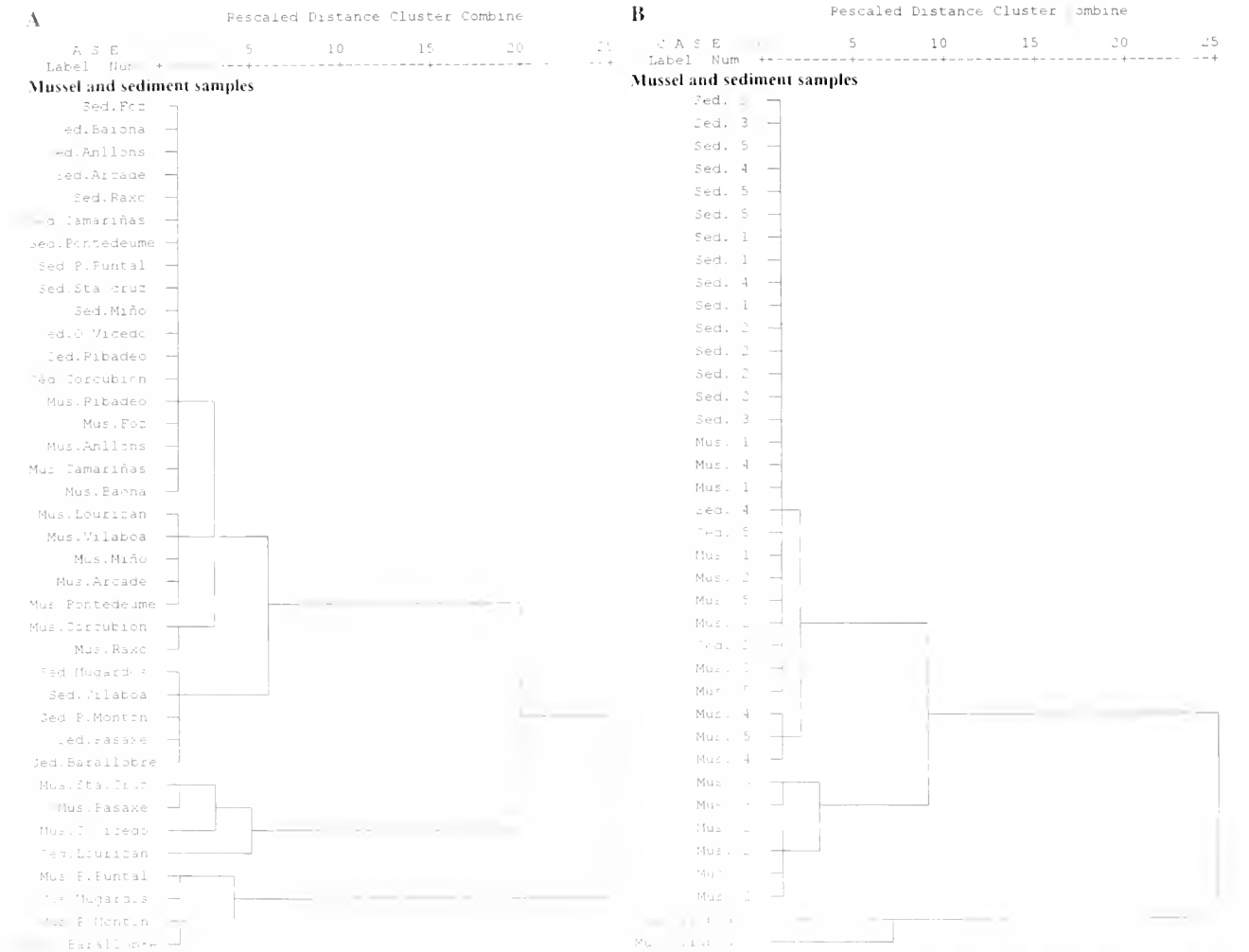

Figure 6. (A) Dendrogram showing the results of a hierarchical cluster analysis on chlorinated biphenyl distributions in Galicia mussels and sediments. (B) Dendrogram showing the results of a hierarchical cluster analysis on lower chlorinated biphenyl distributions in Galicia mussels and sediments.

boa samples (mussels and sediments) were closely grouped (Fig. 6B). The abnormal behavior of the Vilaboa subzone with regard to lower chlorinated biphenyls is probably related to several factors that can cause some special conditions in the aquatic system. The Vilaboa subzone can be considered as an undisturbed area because the presence of strong air and water flows is unlikely, so a major contribution of the highly volatile compounds (with a high vapor pressure) can be caused by atmospheric input. These compounds can be present in sufficient concentrations so that they remain in a free phase and are not dissolved in water.

Sediments from Vilaboa subzone possessed high organic carbon contents. This may influence the fate and bioavailability of some contaminants by affecting physicochemical characteristics, such as water solubility, sorption capacity, fugacity, and partitioning (Kenaga & Goring 1980). It is also known that under anaerobic conditions, microorganisms can partially dechlorinate the more highly chlorinated congeners producing the lower chlorinated compounds (Erickson 1997).

There are reasons to believe that PCB composition will change with time in environmental samples and that the resulting proportions of congeners will not resemble the pattern of industrial mixtures. The lack of definitive assignments of congener composition

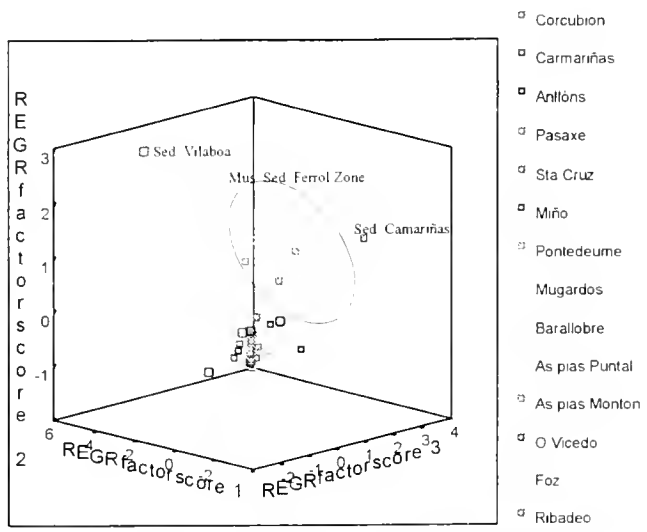

Figure 7. Distribution of Galicia mussels and sediments in the first three principal components (Varimax rotated).

of industrial mixtures and other products contributes to an ignorance of the possible transformations of these compounds in the animal tissues and, in general, in all environmental samples (water, sediment, soil, atmosphere, etc.).

PCA followed the cluster analysis. With the first two factors, 83.46% of initial variance is explained. A representation of the first three rotated (Varimax) components is shown in Figure 7. In the tridimensional space, three sample groups can be identified: the first is closely related to the positive part of the second axis (Vilaboa sediment). The distribution of the second group (mussels and sediments from Ferrol zone, mussels from P. Montón, P. Puntal, Barallobre, and Mugardos and sediments from P. Montón, Pontedeume, Barallobre, and Mugardos) is mainly influenced by the positive part of the first factor. Finally the third group (all the other samples) is related to the negative part of the second axis.

By means of cluster analysis and principal component analysis, the most polluted samples (i.e., the group of Ferrol zone samples of mussels and sediments) and the group of Vilaboa samples (mussels and sediments) were separated from the more heterogeneous group. This study has described the profile and base levels of the organochlorine pollution in estuarine bays from Galicia. In this way, we can now detect abnormal concentrations and possible sources of these compounds that may damage marine water quality and shellfish culture destined for human consumption.

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VALIDITY OF *ESCHERICHIA COLI*, ENTEROVIRUS, AND F-SPECIFIC RNA BACTERIOPHAGES AS INDICATORS OF VIRAL SHELLFISH CONTAMINATION

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ABSTRACT The sanitary classification of harvesting areas for bivalve mollusks in France is based on the level of *Escherichia coli* contamination detected in shellfish meat, as defined in EC Directive 91/492 EEC. However, outbreaks of gastroenteritis or hepatitis after consumption of shellfish meeting current bacteriological standards suggest that *E. coli* is a poor indicator of viral contamination. The purpose of this study was to assess the adequacy of enterovirus and F-specific RNA bacteriophages as new indicators of human enteric viruses. Shellfish were sampled over a 37-mo period to characterize microbial contamination in two coastal areas subjected to different sewage contamination inputs. Contamination by *E. coli*, F-specific RNA bacteriophages (F+ RNA) and human enteric viruses (enterovirus, EV; hepatitis A virus, HAV; Norwalk-like virus, NLV; astrovirus, AV; and rotavirus, RV) was measured in the same samples. *E. coli* analysis was performed by conductance measurement, enteric viruses were detected by reverse-transcription polymerase chain reaction (RT-PCR) and hybridization, and F+ RNA was evaluated by culture according to the ISO 10705-1 method. Statistical analysis based on bootstrap methods was performed on 95 series of paired observations. The validity of *E. coli*, enterovirus, and F-specific RNA bacteriophages as viral indicators was evaluated by measuring their sensitivity and specificity in the presence of enteric viruses. None of the tested indicators proved adequate to protect the public from viral shellfish contamination. The sensitivity of all indicators was better in the highly contaminated zone, and enteroviruses showed the highest specificity for both sites.

KEY WORDS: indicator, *Escherichia coli*, F-specific RNA bacteriophages, enteric viruses, viral contamination, shellfish

INTRODUCTION

Determination of the microbiological sanitary quality of shellfish-growing waters is based on the use of fecal coliforms to evaluate the presence of fecal pollution. In Europe, sanitary criteria are currently established by EC Directive 91/492 EEC. However, previous studies have shown that there is very little relationship between traditional fecal indicators and the presence of viral pathogens (Le Guyader et al. 1994, Doré & Lees 1995, Tree et al. 1997, Lee et al. 1997, Le Guyader et al. 1998). This has been confirmed by outbreaks of viral gastroenteritis or hepatitis after consumption of shellfish meeting current bacteriological standards (Dowell et al. 1995, Lees et al. 1995).

In fact, fecal coliforms are rather poor indicators of viral contamination because they are different than viruses in their resistance to environmental conditions, sewage, or water treatment processes and marine water. They only survive in seawater for a few days, whereas viruses can persist for several months. A few other microbial organisms, such as enteroviruses and bacteriophages, have been proposed as alternative sanitary indicator organisms. As viruses, their survival conditions in seawater should be similar to those of enteric viruses (Havelaar 1993, Metcalf et al. 1995, Maillard 1996, Sobsey 1997). Three main groups of bacteriophages are of particular interest as potential indicators: somatic phages, *Bacteroides fragilis* phages, and F-specific RNA phages. Analysis of existing studies (Lee et al. 1997, Tree et al. 1997, Havelaar et al. 1993) indicated that the last two groups were most suitable for tracing fecal contamination. However, the limited number of *B. fragilis* phages in the environment, as well as methodological difficulties in detecting them, have led some researchers to choose F-specific RNA phages as potential indicators of viral risk in shellfish (Lee et al. 1997). The relation between these indicators and enteric viruses in environmental samples (wastewater, groundwater, fresh and marine waters, and shellfish) is uncertain. Some authors have found a good correlation between these candidate indicators and viruses (Gantzer et al. 1998, Havelaar et al. 1993), whereas others are more doubtful (Legnani et al. 1998, Ricca & Cooney 1999, Griffin et al. 1999, Leclerc et al. 2000).

The purpose of this study was to compare the validity of *E. coli*, enterovirus, and F-specific RNA bacteriophages as viral indicators by measuring the sensitivity and specificity of each candidate in the presence of enteric viruses.

MATERIALS AND METHODS

Environmental Sampling

Shellfish samples were collected over a 3-y period (September 1995 to September 1998) in two coastal areas with different pollution levels. Forty-seven oyster (*Crassostrea gigas*; Thunberg, 1793) samples were collected at three sampling points in a shellfish area temporarily classified as a category A site (site 1) and 48 mussel (*Mytilus galloprovincialis*; Lamarck, 1819) samples at two sampling points in a highly contaminated area not open to shellfish commercialization (site 2). Sample collection was uniform throughout the year: 45% during the autumn-winter season (respectively, 47% in site 1 and 44% in site 2) and 55% during the spring-summer season (respectively, 53% in site 1 and 56% in site 2). Contamination by *Escherichia coli*, F-specific RNA bacteriophages (F+ RNA), and human enteric viruses (enterovirus, EV; hepatitis A virus, HAV; Norwalk-like virus, NLV; astrovirus, AV; and rotavirus, RV) was evaluated in the same samples.

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TABLE 1.

Sensitivity and specificity measurements of an indicator.

	Viral Contamination	
	Yes	No
Positive level of indicator	TP	FP
Negative level of indicator	FN	TN

Sensitivity = TP/(TP + FN). Specificity = TN/(TN + FP).
TP: true-positive; FN: false-negative; FP: false-positive; TN: true-negative.

Microbiological Analyses

E. coli

Quantitative estimation of fecal indicators (*E. coli*) in shellfish was performed by conductance measurements (Dupont et al. 1996). The detection threshold of the conductance method is 30 *E. coli* per 100 g of shellfish (meat and liquor).

Enteric Viruses and Bacteriophages

Upon reception in the laboratory, 10 to 12 shellfish were washed and shucked, and the stomach and digestive tissues were dissected, mixed, aliquoted, and frozen. For analysis, one aliquot was thawed on ice and crushed. Viruses, enteric viruses, and F+ RNA were concentrated by PEG precipitation, as previously described (Atmar et al. 1995).

For human enteric virus detection, the PEG pellet was suspended in phosphate buffer and nucleic acid and purified as previously described (Atmar et al. 1995). All primers, probes, and reverse-transcription polymerase chain reaction (RT-PCR) conditions have already been reported and used in different studies (Le Guyader et al. 2000).

For F-specific RNA bacteriophage enumeration, the PEG pellet was then suspended in 3 mL of peptone water, and F+ RNA phages were detected according to the ISO 10705 (1) 1995 method. The double agar overlay technique was used for preparation and determination of phage stocks and enumeration of the coliphage content of oysters. The F+ RNA assay was performed with host *Salmonella typhimurium* WG49. The detection thresholds of the assay were 2.5 PFU/1.5 g of digestive tissue for oysters and 5 PFU/1.5 g of digestive tissue for mussels.

Data Analysis

Data were first summarized by the minimum, maximum, and geometric mean of the shellfish concentration for *E. coli* and F+ RNA and by the presence or absence of detection for enteric viruses (EV, HAV, NLV, AV, and RV). The validity of an indicator organism to discriminate shellfish samples contaminated by virus was evaluated using the sensitivity of each candidate indicator organism for virus detection. The accuracy of identifying samples with no viral contamination was estimated by the specificity of the indicator.

Sensitivity was defined as the ratio of the number of true-positive values (positive level of the candidate indicator organism in the presence of virus) to the number of true-positive values plus the number of false-negative values (negative level of the candidate indicator organism in the presence of virus). Specificity was defined as the ratio of the number of true-negative values (negative level of the candidate indicator organism in the absence of virus) to the number of true-negative values plus the number of false-positive values (positive level of the candidate indicator organism in the absence of virus) (Table 1). Sensitivity and specificity estimates ranged between 0 and 1 (or 0% and 100%). Ideally, the best indicator should have a sensitivity and specificity of 1 (or 100%).

An indicator was considered to be positive or negative according to a specific threshold. The negative level of the threshold was less than 230 *E. coli* per 100 g of shellfish meat for *E. coli*, the detection level for F+ RNA and absence of detection for EV. The positive level of the threshold was equal to or greater than 230 *E. coli* per 100 g of shellfish flesh for *E. coli*, above the detection level for F+ RNA and positive detection for EV.

The variance estimators for sensitivity and specificity were evaluated using resampling techniques (Efron 1982). Resamples were generated from a nonparametric bootstrap procedure based on the initial samples, consisting of 47 pairs of data for site 1 and 48 pairs of data for site 2 for each organism. Each pair related to the concentration of the organism (*E. coli*, F+ RNA) or the presence/absence of the organism (EV), or the presence/absence of the virus. One thousand replicate samples were generated for each organism based on these data. The mean and variance of both sensitivity and specificity were calculated for the three indicator organisms.

Two conditions were tested for viral contamination. The first concerned all the enteric viruses (V1, i.e., HAV and/or NLV and/or AV and/or RV) except EV, which was used here as an indicator. The other focused on viruses known to be involved in shellfish-borne viral disease (V2, i.e., HAV and NLV). For each condition, a *t*-test at a 95% confidence level was used to compare differences in mean sensitivity and mean specificity between two indicators. Statistical analyses were performed using S-Plus 2000 (MathSoft, Inc., Seattle, WA).

RESULTS

The results obtained throughout the study period for *E. coli* and F+ RNA are shown in Table 2. At site 1, *E. coli* concentrations in shellfish ranged from <30 to 5,670 per 100 g of shellfish, with a geometric mean of 55. At site 2, the maximum value and geometric mean were 20,200 and 283 *E. coli* per 100 g. The F+ RNA content in shellfish ranged between <2.5 and 87 phages per 1.5 g

TABLE 2.

Concentrations (minimum, maximum, and geometric mean) of *E. coli* and F+ RNA in shellfish collected in the study areas.

		E. coli (/100 g of Shellfish)			F+ RNA (/1.5 g of Digestive Tissue)		
	n Analyzed Samples	Min.	Max.	Geometric Mean	Min.	Max.	Geometric Mean
Site 1	47	<30	5,670	55	<2.5	87	3.9
Site 2	48	<30	20,200	283	<5	4,12	46

of digestive tissue with a geometric mean of 3.9 phages per 1.5 g at site 1. Bacteriophage contamination was greater at site 2, with a minimum and maximum of <5 and 4,125, phages per 1.5 g and a geometric mean of 46 phages per 1.5 g. The minimum values obtained at sites 1 and 2 corresponded to the detection limits of the analytical methodology. All enteric viruses except hepatitis A virus at site 1 were found in sampling areas (Table 3). Shellfish were contaminated more often by all types of viruses at site 2 than at site 1.

All *t*-tests comparing mean sensitivity and mean specificity were significant except, at site 1, for the sensitivity results between *E. coli* and EV for V2 (viruses involved in shellfish-borne viral disease, i.e., HAV and VNL). At site 1 (Table 4), F+ RNA had low sensitivity measurements of 0.23 for V1 (all enteric viruses) and 0.50 for V2. This potential viral indicator was associated with viral contamination in only one of five samples for V1 and one of two for V2. The results obtained for *E. coli* and EV sensitivity were lower (0.09 for *E. coli* and 0.13 for EV for V1, and both 0.20 for V2). For V2, the difference in sensitivity between F+ RNA and the other indicators was particularly striking. For specificity, enteroviruses gave the highest estimates (0.92 for V1 and V2) compared to *E. coli* (0.80 for V1, 0.86 for V2) and F+ RNA (0.84 for V1; 0.89 for V2).

The adequacy of the viral indicator relative to viral contamination was greater at site 2 (Table 5) than site 1. Sensitivity estimates ranged between 0.57 (EV) and 0.80 (F+ RNA) for V1 and between 0.73 (EV) and 1.00 (F+ RNA) for V2. The sensitivity of *E. coli* was intermediate, (i.e., 0.66 for V1 and 0.80 for V2). Enterovirus specificities were highest (1.00 for V1 and 0.82 for V2), whereas F+ RNA had the lowest capacity to identify noncontaminated shellfish samples (specificity estimates of 0.33 for V1 and 0.37 for V2). The specificity estimates of *E. coli* were between those calculated for EV and F+ RNA (0.55 for V1 and 0.52 for V2). The sensitivity and specificity estimates for a given candidate were statistically different from those obtained for the others, according to V1 or V2 (*t*-test, 5% in Table 5).

DISCUSSION

The method applied here is currently being used in clinical experiments (Désenclos et al. 1997) to evaluate the accuracy (sensitivity and specificity) of a new screening procedure compared to a gold standard. Sensitivity represents the ratio of individuals classified as "ill" by the method among those known to have the disease, and specificity represents the ratio of individuals classified as "not ill" among those known not to have the disease. These criteria are calculated according to a threshold, and the relative frequency of true-positive (TP), false-negative (FN), false-positive (FP), and true-negative (TN) results depends on the level of this threshold. A low threshold increases sensitivity and reduces specificity, and the opposite is true for a high threshold. The diagnostic strategy requires that sensitivity or specificity be concordant with the objective (avoiding false-negative or false-positive results).

In environmental research, this method was used to compare the validity of *E. coli*, EV and F+ RNA as viral indicators. The purpose of sanitary analysis of shellfish is to provide guarantees that protect consumers from public health hazards due to the consumption of polluted shellfish. In this context, the sensitivity of an indicator is the prime criterion. In other respects, the specificity of an indicator has commercial implications, limiting the period of temporary closure of a shellfish area because of public health concerns. Moreover, sensitivity measurements have greater public health implications in shellfish-growing areas classified as category A sites since these shellfish are consumed directly (without any purification treatment). Accordingly, the following comments are concerned mainly with sensitivity criteria and focused on results obtained for site 1.

No indicator was highly sensitive to viral shellfish contamination in samples from the slightly contaminated area (site 1). Results for F+ RNA sensitivity, though better than those for the other indicators, were not fully predictive of the viral risk related to shellfish consumption. Classification errors were numerous: 50% of the results were FN for viruses involved in shellfish-borne viral disease, V2 (80% FN for all enteric viruses, V1). F+ RNA sensitivity could not be improved because the tested threshold was the detection level of the F+ RNA analytical method (2.5 PFU/1.5 g of digestive tissue for oysters and 5 PFU/1.5 g of digestive tissue for mussels).

TABLE 3.

Detection of viruses in shellfish collected in the study area (a sample may have been contaminated by more than one virus).

	n Analyzed Samples	EV (*n*)	HAV (*n*)	NLV (*n*)	AV (*n*)	RV (*n*)
Site 1	47	5	0	10	9	11
Site 2	48	17	6	12	20	19

EV: enterovirus; HAV: hepatitis A virus; NLV: Norwalk-like virus; AV: astovirus; RV: rotavirus.

TABLE 4.

Sensitivity and specificity measurements for each indicator organism in site 1 ($n = 47$).

	V1		V2	
Site 1	Sensitivity	Specificity	Sensitivity	Specificity
E. coli	0.09	0.80	0.20	0.86
EV	0.13	0.92	0.20	0.92
F+ RNA	0.23	0.84	0.50	0.89

V1 represents the presence or absence of HAV and/or NLV and/or AV and/or RV; V2 represents the presence or absence of viruses involved in shellfish-borne viral disease (HAV and NLV). All results were significantly different from others (*t*-test, 5%), except for the two 0.20-obtained for V2.

TABLE 5.

Sensitivity and specificity measurements for each indicator organism in site 2 ($n = 48$).

	V1		V2	
Site 2	Sensitivity	Specificity	Sensitivity	Specificity
E. coli	0.66	0.55	0.80	0.52
EV	0.57	1.00	0.73	0.82
F+ RNA	0.80	0.33	1.00	0.37

V1 represents the presence or absence of HAV and/or NLV and/or AV and/or RV; V2 represents the presence or absence of viruses involved in shellfish-borne viral disease (HAV and NLV). All results were significantly different from others (*t*-test, 5%).

The inadequacy of *E. coli* standards for public health protection was confirmed in this study. *E. coli* was the least sensitive and specific tool for identifying samples contaminated by enteric viruses. Enterovirus was not better than *E. coli* in predicting a public health risk, but showed a high degree of accuracy in predicting the absence of viruses, with a low error classification (8% FP). For sporadic contamination, the tested indicators (*E. coli*, EV, and F+ RNA) were not sensitive to the targeted viruses, either because the epidemic cycles were different or because the behavior and survival of the viruses in seawater were variable. Although *E. coli* is part of the bacterial flora of warm-blooded animal feces, F+ RNA phages are an infrequent component of feces. However, these phages are abundant in sewage treatment plants where they proliferate at low temperatures. As they are resistant to various sewage treatment processes and to chlorination, they are more suitable for tracing a sewage effluent than indicating fecal pollution (Havelaar & Pot-Hogeboom 1988, Leclerc et al. 2000). The presence of enteric viruses in sewage water and eventually in coastal water and shellfish is correlated with viral diseases in the general population. Epidemics of viral diarrhea with NLV, RV, and AV etiologies usually follow a seasonal pattern, with a peak in winter (Kapikian et al. 1996, Vinjé et al. 1997, Desselberger 1998, Evans et al. 1998, Otsu 1999). Outbreaks of hepatitis A can occur throughout the year, depending on the different exposure conditions (i.e., contaminated food and water, contact with sewage, travels in endemic countries, direct human transmission) (Hollinger & Ticehurst 1996). Pathologies caused by enteroviruses occur most frequently in summer (Melnick 1996). These epidemiological aspects indicate the difficulty of finding a universal viral indicator.

The sensitivity of all indicators tested was better for samples from the contaminated zone (site 2) than from site 1. As this area is highly affected by urban sewage and is contaminated year-round by fecal coliforms and F+ RNA, these potential indicators could be present systematically when enteric viruses in effluent sewage are discharged into the marine environment during human viral gastroenteritis epidemics. Thus, F+ RNA sensitivity measurements could reach 100%. However, as this area is strictly forbidden for shellfish commercialization on the basis of *E. coli* criteria, a viral indicator would not be more satisfactory than fecal coliforms. These findings are consistent with those already published on marine water contamination in highly contaminated areas (Chung et al. 1998, Legnani et al. 1998).

Concerning areas in compliance with microbiological sanitary criteria, our results differ from those in previous reports recommending F+ RNA as a viral indicator (Chung et al. 1998, Doré et al. 2000), probably because of variations in F+ RNA contamination levels. Moreover, one of these studies (Doré et al. 2000) concerned the use of F+ RNA to evaluate the sanitary quality of market-ready oysters after purification (generally 48 h according to regulations) and not, as in our study, to monitor the water quality in shellfish harvesting areas. During the purification process, the *E. coli* concentration in shellfish is drastically reduced within a 48-h period as compared to phage and virus levels. This short purification period may not be long enough to eliminate viral contamination (Schwab et al. 1998). In such conditions, F+ RNA phages should be reliable indicators of enteric viruses in oysters (Doré et al. 2000).

In a long-term environmental survey, the occurrence and concentration of F+ RNA, as compared to enteric viruses, would probably differ throughout the year. This would be particularly true for slightly contaminated areas in which sewage discharge is sporadic. The F+ RNA results observed in the present study are directly comparable with those of enteric virus detection because both relate to the analysis of digestive gland in which enteric viruses and F+ RNA are mainly concentrated (Atmar et al. 1995, Doré & Lees 1995). Similarly, Croci et al. (2000) found no correlation between enteroviruses, hepatitis A virus, bacteriophages, and *E. coli* in mussels collected in waters in accordance with microbiological sanitary criteria. These authors, as well as Toze (1999), recommended that viruses be detected directly in shellfish to prevent health risks related to their consumption.

Our results indicate that the traditional and proposed indicators tested did not fully prevent the viral risk related to shellfish consumption, especially in slightly contaminated shellfish-growing areas. Direct detection of the real pathogen by molecular tools could provide another means of assessing viral risk for shellfish consumers. This recommendation needs to be considered during high-risk seasons when enteric viruses are likely to be present in the population and enter into wastewater and sewage subsequently discharged into the marine environment (Burkhardt & Calci 2000, Miossec et al. 2000). Further studies are required to confirm these findings.

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NATURAL OCCURRENCE OF *VIBRIO* SPP. AND *LISTERIA MONOCYTOGENES* IN MOLLUSCAN SHELLFISH IN PORTUGAL

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ABSTRACT The natural occurrence of *Vibrio* spp. and *Listeria monocytogenes* in shellfish was analyzed over a period of 18 months. Sixty-one samples of 14 different species of molluscan shellfish were collected in local Lisbon markets (Portugal). *Vibrio* species were detected in 28 shellfish samples (45.9%). Different vibrio species were identified in varying percentages of the shellfish samples tested: *V. parahaemolyticus* (11.4%), *V. alginolyticus* (6.5%), *V. vulnificus* (22.9%), *V. damsela* (9.8%), *V. metschnikovii* (1.6%), and *Vibrio spp.* (21.3%). *Listeria monocytogenes* was isolated from eight samples (13.1%). Other *Listeria* species, *L. innocua* and *L. grayi*, were found in 3.3% and 1.6% of shellfish samples, respectively. *L. monocytogenes* was found simultaneously with other different *Listeria* species in two samples. All *L. monocytogenes* isolates were anti-sera poly O 1–4+. Five samples (8.2% of total) were simultaneously contaminated with *Vibrio* spp. and *Listeria* spp. These results show that *Vibrio* spp. and *L. monocytogenes* are frequently found in shellfish products approved as safe, and the public health significance of these results should not be overlooked.

KEY WORDS: *Vibrio* spp., *Listeria* spp., Portugal, occurrence

INTRODUCTION

Seafood has traditionally been a popular part of the diet in many regions of the world and in some countries, such as Japan, constitutes the main supply of animal protein. Today even more people are turning to fish as a healthy alternative to red meat (Koenig et al. 1991), although there is still some resistance to this habitude. For example, between 1973 and 1987 in the United States, the meat consumption was approximately 10 times that of fish (Huss 1994). This tendency of considering fish as healthy is related to the low fat content of many fish species and the effects on coronary heart disease of the W-3 polyunsaturated fatty acids found in fatty fish species, which are extremely important where cardiovascular disease mortality is high. However, consumption of fish and shellfish has been implicated in many cases of food-borne diseases outbreaks (Huss 1994).

The various disease agents that have been associated with the consumption of seafood may conveniently be divided into two groups: indigenous bacteria and nonindigenous bacteria (Kueh & Chan 1985). *Vibrio* spp. and *Listeria* spp. are included in the mentioned group first (Huss 1994).

Pathogenic *Vibrio* spp. are a human health hazard that occur naturally in estuarine waters. They are frequently found in filter-feeding molluscan shellfish, which are generally the source of infection. Disease resulting from raw oyster ingestion results in fatality rates exceeding 50% for *V. vulnificus* infections (Wittman & Flick 1995, Hlady 1997). The incidence of pathogenic *Vibrio* spp. has been correlated with elevated water temperatures (Kaneko & Colwell 1973, Hlady 1997). Typically, pathogenic *Vibrio* spp. multiply rapidly at temperatures between 20°C and 40°C. This is reflected in the large numbers of the organisms isolated from molluscan shellfish when water temperature rises to 30°C, and their virtual absence from molluses taken from cold waters (ICMSF 1998). For example, *V. parahaemolyticus* is widespread when water temperatures exceed 15°C (Kaneko & Colwell 1973). Nevertheless they also have been isolated from crabs taken from cold waters (ICMSF 1998). *Vibrio* spp. levels in the estuarine environment are also dependent on the time of the day, depth, and tidal levels, and each of those factors must be taken into account to get an accurate estimate of vibrios in the environment (ICMSF 1998). *V. parahaemolyticus*, *Vibrio cholerae*, and *Vibrio vulnificus* are routinely part of the microflora in crustaceans captured from estuarine waters. *Vibrio* species cause a variety of human infections, which can usually be classified as gastrointestinal or extraintestinal infections (Kudoh 1988). Species most commonly associated with diarrhea or gastroenteritis include *V. cholerae* (O1 and non-O1) and *V. parahaemolyticus*. However, *V. fluvialis*, *V. furnissii*, *V. mimicus*, and *V. hollisae* have also been shown to cause or be significantly associated with gastroenteritis (Kudoh 1988, Wittman & Flick 1995). Interestingly, some of these *Vibrio* species may also cause extraintestinal infections such as wound infections and secondary septicemia (Kudoh 1988). *V. vulnificus* is the well-known cause of primary and/or secondary septicemias and wound infections with high mortality. The hazards associated with ingestion of seafood contaminated with this potential pathogen have resulted in a precautionary statement issued by the U.S. Food and Drug Administration against consumption of raw or undercooked seafood by people with hepatic disorders (Anonymous 1992). *V. alginolyticus* and *V. damsela* also cause several types of soft-tissue infections and wound infections. *V. metschnikovii* has been implicated as a cause of opportunistic infections, but its pathogenic significance in the illness needs further investigation. According to Wittman and Flick (1995), between 1984 and 1993, the greatest percentage of known death (95%) due to shellfish-borne disease was caused by noncholera vibrios; noncholera vibrios also accounts for the second highest causative agents of disease.

Fish and seafood are considered potential sources of *Listeria* spp. because this microorganism is ubiquitous in nature (Farber & Peterkin 1991) and salt-tolerant (Dillon & Patel 1992). Fisheries and aquaculture products are directly exposed to water contamination. In fact, *Listeria* spp., and particularly *L. monocytogenes*, have been isolated from a variety of fish and shellfish products (Ben Embarek 1994, Dillon et al. 1992, Dillon et al. 1994, Jørgensen & Huss 1998, Weagant et al. 1988). Although not very involved in listeriosis, the presence of *L. monocytogenes* in seafood and particularly in shellfish has been implicated in some cases. A described epidemic of perinatal listeriosis was related to the consumption of raw fish and shellfish (Lennon et al. 1984), and

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some sporadic cases have been identified as associated with shellfish, precisely smoked mussels and raw oysters (Plusquellec et al. 1998). Also a small outbreak of listeriosis associated with smoked mussels has been reported (Brett et al. 1998). To minimize bacterial growth and exposure to this organism, it is important to have adequate control of the environment for food production as well as to take precautions for adequate cooking and handling food (Roberts 1994).

In Portugal, like in many other countries, food-borne diseases are not appropriately reported, thus the epidemiological importance of seafood-borne diseases is lacking. Although some references have been made on the isolation of *L. monocytogenes* in molluscan shellfish in Portugal (Pedro 1996), there is not much information on this subject and the occurrence of *Vibrio* spp. is totally unknown. The consumption of molluscan shellfish is very popular in Portugal, and has increased in the last ten years by 4% (SNI 1997). Additionally, raw or undercooked molluscan shellfish are the favored ways of eating it. The aim of this work was to perform an evaluation of the occurrence of *Vibrio* spp. and *Listeria* spp., particularly *L. monocytogenes*, in molluscan shellfish commercialized in Portugal to obtain some information about the significance of these hazards.

MATERIAL AND METHODS

Samples were collected between March 1998 and July 1999. Fresh molluscan shellfish were obtained from local Portuguese markets and were investigated for the presence of *Vibrio* spp. and *Listeria* spp. A total of 61 samples included three oysters (*Crassostrea angulata*), one hen clam (*Mactra coralina*), five furrow shell (*Scrobicularia plana*), seven mussels (*Mytilus edulis*), seven wedge shell (*Donax trunculus*), six carpet shell (*Tapes decussatus*), seven purr (*Veneropsis corrugata*), two purr (*Veneropsis rhomboide*), seven common cockle (*Cerasthoderma edule*), four periwinkles (*Litorrina littorea*), four whelk (*Buccinnum* sp.), four razor shell (*Solen marginatus*), two cuttlefish (*Sepia officinalis*), and two barnacles (*Pollicipes cornucopia*). All samples were kept below 5°C until arrival at the laboratory, then immediately analyzed. Fifty grams of each sample were suspended in 450 mL of distilled water with 3% NaCl (Merck, Art. 6404) and homogenized for 1 min in a Stomacher (Lab-Blender 400).

For *Vibrio* research, molluscan shellfish were previously washed with a volume of water that was twice its volume in order to detect outer side contamination (shell contamination). This washing water and the homogenized samples were platted on thiosulphate citrate bile sucrose agar (TCBS) (Oxoid CM333) and incubated at 37°C for 24 h to determine the levels of *Vibrio* spp. without a previous enrichment. A selective enrichment was also performed in alkaline peptone water (Oxoid CM9), incubated at 37°C for 24 h, followed by plating on TCBS. After incubation at 37°C for 24 h, the presumptive colonies, sucrose-negative and sucrose-positive, were confirmed by their sensibility to the vibriostatic compound 0:129 (Oxoid DD14 and DD15) and biochemically identified by Api 20NE (Bio-Mérieux 20050) and Api 20E (Bio-Mérieux 20100). Colonies not identified to species were designated *Vibrio* spp.

Isolation of *Listeria* spp. was performed using a nonselective enrichment broth, buffered peptone water (BPW) (Oxoid CM 509). After 24 h of incubation at 30°C, 1 mL was transferred to both 10 mL of enrichment Fraser Broth (Merk 110398) and thioglycollate medium U.S.P. (Oxoid CM173). A selective enrichment procedure was performed after 24 h at 30°C by transferring 1 mL of each previous enrichment to both 10 mL of University of Vermont Medium with supplement 1 (UVM I) (Oxoid CM863 and SR142) and Fraser Broth (Merk 110399) with half-strength supplement (Merk 110399).

Plating on selective medium, Palcam agar (Oxoid CM8719, SR150E) and McBride agar (Difco 0922-17), followed both nonselective and selective enrichment procedures after incubation at 30°C for 24 h and 30°C for 48 h, respectively. Palcam agar plates were incubated at 30°C for 24–48 h and McBride agar plates at 37°C for 24–48 h. Four or five presumptive colonies of each plate were subcultured for purity on Tryptone-Soya Agar (Oxoid CM131) with 0.6% of yeast extract (Difco 0127-01-7).

Identification of the isolates was performed using conventional tests: catalase and oxidase test, Gram reaction, and observation of bacterial morphology. Tumbling motility at 25°C was observed, and respiratory type and umbrella motility were tested on SIM medium (Oxoid CM435). CAMP test and the production of β-hemolysis was performed on Columbia agar plus 5% sheep-blood (BioMérieux 43 041). Api *Listeria* strips (BioMérieux 10 300) were used for biochemical identification (Fig. 1). Serological tests were performed using *Listeria* O antiserum poly serotypes 1, 4 (Difco Laboratories, Detroit, MI).

RESULTS

Vibrio spp. were isolated from 31 of the 61 samples (50.8%). From the 14 different species of molluscan shellfish studied (Table 1), *Vibrio* spp. were found in all species, except one, *Mactra coralina*. Five different species of *Vibrio* were identified: *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. damsela*, and *V. metschnikovii*. *V. parahaemolyticus* was found in *M. edulis*, *T. decussatus*, *V. corrugata*, *V. rhomboide*, *C. edule*, *L. littorea*, and *Buccinum sp.* *V. vulnificus* was found in *C. angulata*, *M. edulis*, *D. trunculus*, *T. decussatus*, *V. corrugata*, *C. edule*, *L. littorea*, *Buccinum* sp., *S. marginatus*, *S. officinalis*, and *P. cornucopia*. *V. alginolyticus* was found in *D. trunculus*, *C. edule*, *Buccinum* sp., and *S. officinalis*. *V. damsela* was found in *D. trunculus*, *C. edule*, *Buccinum* sp., *S. marginatus*, and *S. officinalis*. *V. metschnikovii* was found in *V. corrugata* (Table 2). The highest levels found were for *V. parahaemolyticus* ($10^{2.3}$ cfu/g) in *V. rhomboide* and *L. littorea*, and *V. alginolyticus* ($10^{2.3}$ cfu/g) in *D. trunculus* and *C. edule*, followed by *V. metschnikovii* (10^{2} cfu/g) and *V. vulnificus* ($10^{1.8}$ cfu/g), both in *V. corrugata*. The highest levels of contamination were found in the inner tissue of the shellfish (Table 2).

V. vulnificus was found all through the year, even during the cold months of winter (November, January, and February). On the other hand, *V. parahaemolyticus* was only found in warmer months of May, June, and October. *V. alginolyticus* and *V. damsela* were also found all through the year (Fig. 1).

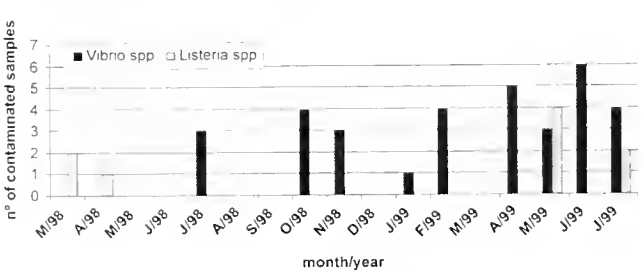

Figure 1. Number of samples contaminated with *Vibrio* spp. and *Listeria* spp. in monthly samples: March 1998 to July 1999.

TABLE 1.

Frequency of *Vibrio* spp. and *L. monocytogenes* in Portuguese molluscan shellfish.

Shellfish Species	*n*	*Vibrio* spp. N+ (%)	*Listeria* spp. N+ (%)	*Vibrio* spp. and *Listeria* spp. N+ (%)
Crassostrea angulata	3	1	0	0
Mactra coralina	1	0	1	0
Scrobicularia plana	5	1	1	0
Mytilis edulis	7	2	2	1
Donax trunculus	7	3	0	0
Tapes decussatus	6	4	0	0
Veneropsis corrugata	7	2	0	0
Veneropsis rhomboide	2	1	0	0
Cerasthoderma edule	7	5	2	2
Litorrina littorea	4	3	0	0
Buccinun sp	4	2	1	1
Solen marginatus	4	4	1	1
Sepia officinalis	2	2	0	0
Pollicipes cornucopia	2	1	1	0
Total	61	31 (50.8%)	9 (14.8%)	5 (8.2%)

Listeria spp. was isolated in 9 of the 61 samples (14.8%) (Table 1). The contaminated samples were: *C. edule* (two), *M. edulis* (two), *M. coralina* (one), *S. plana* (one), *P. cornucopia* (one), *S. marginatus* (one), and *Buccinum* sp. (one). Different species of *Listeria* were isolated. *L. monocytogenes* was the most frequent, found in eight samples (13.1%), whereas *L. innocua* was found in two samples (3.3%) and *L. grayi* in one sample (1.6%) (Table 3). *L. monocytogenes* was found concomitantly with other *Listeria* species in two samples, once with *L. innocua* and *L. grayi* in *M. coralina* and once with *L. innocua* in *M. edulis*. All *L. monocytogenes* isolates were anti-sera poly O 1–4+. Five samples (8.2%) were simultaneously contaminated with *Vibrio* spp. and *Listeria* spp. (Table 1).

DISCUSSION

Since *Listeria* spp. are ubiquitous and *Vibrio* spp. are naturally occurring in the aquatic environment, their reported prevalence is not surprising. Moreover, shellfish filter large quantities of water and therefore are able to concentrate water-borne pathogens including some *Vibrio* spp. and *L. monocytogenes* (NACMCF 1992).

The *Vibrio* spp. levels associated with the outer side of the shellfish suggests that shellfish manipulation as well as cross-contamination with *Vibrio* can also be a hazard (Hobbs & Roberts 1987). Contamination levels show that *Vibrio* spp. can be an important hazard in molluscan shellfish safety because many outbreaks are due to consumption of raw seafood that may be naturally contaminated with small numbers of *Vibrio* spp. but are held unrefrigerated long enough to allow organisms to proliferate to large numbers. Outbreaks involving boiled shellfish have been attributed to failure to cook the shellfish at temperatures high

TABLE 2.

Vibrio species and levels of contamination (cfu/g) in Portuguese shellfish and associated with the outside of the shells (cfu/g).

			Vibrio Species											
			Vibrio parahaemolyticus		*Vibrio vulnificus*		*Vibrio alginolyticus*		*Vibrio damsela*		*Vibrio metschnikovii*		*Vibrio* spp.	
Sample	*n*	*Vibrio* spp N+ (%)	In	Out	In	Out	In	Out	In	Out	In	Out	In	Out
C. angulata	3	1			$10^{-0.7}$									
M. coralina	1	0												
S. plana	5	1											$10^{1.3}$	$10^{1.3}$
M. edulis	7	2	$10^{1.3}$ $10^{1.3}$			$10^{1.6}$							$10^{-0.7}$	
D. trunculus	7	3			$10^{0.25}$	$10^{0.3}$	$10^{2.3}$	$10^{0.3}$	$10^{-0.7}$					$10^{-0.7}$ $10^{0.3}$
T. decussatus	6	4		$10^{0.3}$ $10^{-0.7}$ $10^{0.3}$	$10^{0.3}$	$10^{0.6}$								$10^{-0.7}$ $10^{0.3}$ $10^{1.3}$ $10^{1.3}$
V. corrugata	7	2	$10^{0.3}$	$10^{-0.7}$		$10^{1.8}$ 10^{1}					10^{2}			
V. rhomboide	2	1	$10^{2.3}$											$10^{1.3}$
C. edule	7	5	$10^{0.3}$		$10^{-1.2}$ $10^{0.3}$	$10^{-0.7}$ $10^{0.3}$ $10^{0.3}$	$10^{0.3}$ $10^{2.3}$		$10^{-0.7}$				$10^{-0.7}$	$10^{-0.7}$
L. littorea	4	3	$10^{2.3}$	$10^{1.3}$	$10^{1.3}$									$10^{1.8}$ $10^{-0.7}$ $10^{-0.7}$
Buccinum sp.	4	2	$10^{1.3}$		$10^{-0.7}$	$10^{0.5}$	$10^{1.3}$			$10^{1.3}$			$10^{-0.7}$	10^{1}
S. marginatus	4	4				$10^{0.3}$			$10^{2.7}$					$10^{0.3}$
S. officinalis	2	2				$10^{-0.7}$		$10^{-0.7}$		$10^{1.3}$				$10^{-0.7}$ $10^{-0.7}$
P. cornucopia	2	1				$10^{1.3}$								

TABLE 3.

Occurrence of *Listeria* spp. in molluscan shellfish samples.

Sample	*n*	N+ (%)	N+/*Listeria* Found Species		
			monocytogenes	*innocua*	*grayi*
C. ongulata	3	0	0	0	0
M. coralina	1	1	1	1	1
S. plana	5	1	1	0	0
M. edulis	7	2	1	1	0
D. trunculus	7	0	0	0	0
T. decussatus	6	0	0	0	0
V. corrugata	7	0	0	0	0
V. rhomboide	2	0	0	0	0
C. edule	7	2	2	0	0
L. litorea	4	0	0	0	0
Buccinum spp.	4	1	1	0	0
S. marginatus	4	1	1	0	0
S. officinalis	2	0	0	0	0
P. cornucopia	2	1	1	0	0
Total	61	9 (14.8%)	8 (13.1%)	2 (3.3%)	1 (1.6%)

enough to kill *Vibrio* spp. Other outbreaks have been attributed to contamination of cooked seafood by raw seafood followed by inadequate refrigeration (Blake et al. 1980, Hobbs & Roberts 1987). The levels from the inner tissue of the animal are lower than the ones reported previously from molluscan shellfish collected in Hong Kong markets that ranged between 10^4 and 10^5 cfu/g (Chan et al. 1989). This might be due to the lower temperatures of the seawater in Europe (between 13°C in winter and 22°C in summer). On the other hand, it is known that enrichments plated on CPC agar are more appropriate for *V. vulnificus*, as well as including the addition of polimixin to the enrichment medium (Dalsgaard & Hoi 1997). This might explain the lower levels obtained once the initial research procedures were performed with nonspecific culture media. However, media designed for one vibrio could delay or inhibit the growth of other *Vibrio* species. The incidence of *V. vulnificus*, *V. alginolyticus*, and *V. damsela* was not characterized by a seasonal distribution, probably due to the low temperature amplitudes in Portuguese seawater. On the other hand, *V. parahaemolyticus* was isolated only during the warmer months.

Listeria-contaminated samples were collected on March and April 1998 and May and July 1999 (Fig. 1), which reveals the tendency of this microorganism to develop in warmer temperatures (Dillon and Patel 1992). In a previous study conducted in Portugal, Pedro (1996) isolated *Listeria* spp. and *L. monocytogenes* in 23% and 20%, respectively, of samples from 35 shellfish. Isolations were only performed on clams and furrow shells. These results and this study's results obtained in Portugal reveal some differences when compared with those cited in literature where the presence of this microorganism is mostly found in oysters and mussels. de Simón et al. (1992) found *Listeria* spp. in 22.5% of the 40 samples of mussels analyzed and detected *L. monocytogenes* in three of them (7.5%). These authors also isolated *L. innocua* and *L. seeligeri* in 12.5% and 2.5% of the mentioned samples, respectively. In oysters, Weagant et al. (1988), Buchanan et al. (1989), and Colburn et al. (1990) did not find *Listeria* in any seafood sample. Motes (1991) did not find *Listeria* spp. from 75 oysters samples, all collected from estuarine environments along the U.S. Gulf Coast. Wilson (1995) isolated *Listeria* spp. in 11.0% of the molluscan/crustacean samples analyzed (8/74) in Ireland. In Finland, Hartemink and Georgsson (1991) found *Listeria* spp. in samples of raw and smoked fish, but no isolation was made in 11 shellfish samples. Jeyasekaran et al. (1996) isolated *Listeria* spp. in 44.4% (16/36) of shellfish samples in India, and *L. monocytogenes* was found in 12.1% of the contaminated samples. These authors also isolated *L. innocua* and *L. welshimeri* in 36.1% and 5.6% of the tropical samples, respectively.

In our study, *L. monocytogenes* was the predominant species (13.1%) and *L. innocua* the second most common (3.3%). In the literature *L. innocua* is seldom referred to as being the most commonly isolated species (Weagant et al. 1988, Ryu et al. 1992, Wilson 1995, Jeyasekaran et al. 1996). This fact is related to the shorter generation time of *L. innocua* in enrichment broth, which may lead to false-negative *L. monocytogenes* results in cultures where the two species are both present (Petran & Swanson 1993, Curiale & Lewus 1994). On the other hand, the coexistence of these two species in some samples (Table 3) indicates that these microorganisms may share the same ecological niche. Furthermore, the presence of nonpathogenic *Listeria* spp. is considered to be an "indicator" of the presence of the pathogenic species (King et al. 1989). The nonselective enrichment step followed by a selective enrichment in two different media might have contributed to the slight enhancement of *L. monocytogenes*, because it constitutes a resuscitation step that may favor its recovery. This procedure has been recommended especially when examining products where *Listeria* spp. cells are expected to be injured (McCarthy et al. 1990).

Vibrio spp. and *Listeria* spp. were isolated simultaneously in five samples. These results have never been reported in Portugal. The analysis of the epidemiology of some food-borne diseases reveals a significantly higher rate of coinfection in patients due to naturally occurring pathogens (Hlady 1997). Hence, these results suggest the possibility of a higher risk with the consumption of these particularly molluscan shellfish.

Considering the direct utilization of molluscan shellfish in human diet and the deficient cooking habits applied to shellfish, *L. monocytogenes* and *Vibrio* spp. occurrence must be regarded as a relevant hazard to public health. Therefore, due to the importance of seafood on the Portuguese alimentary habits, it would be important to establish the real hazards to humans.

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THE PREVALENCE OF NONCULTIVABLE BACTERIA IN OYSTERS (*TIOSTREA CHILENSIS*, PHILIPPI, 1845)

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ABSTRACT Genotypic analysis based on molecular methods has become a useful tool to determine the composition of bacterial populations and communities. We used this tool to study the microflora of oysters. Analysis of the electrophoretic mobility of the 16S–23S rDNA spacers of the bacteria, isolated from oysters and cultured in marine agar, revealed the presence of three main groups of bacteria. Within each group the bacterial strains had identical 16S rDNAs, as judged by the electrophoretic mobility of the heteroduplexes formed between amplified 16S rDNA. The extrapolation of these findings to the total bacterial population, however, seems unwarranted because the number of colonies grown in agar was less than 0.001% of the bacteria present in the oysters. Consistently, the electrophoretic mobility of the 16S–23S rDNA spacers of the total bacterial DNA extracted from oysters had a very different pattern of that extracted from the bacterial cultures. Analysis of bacterial DNA obtained from individual oysters showed the presence of some spacers in most of the oysters examined. These spacers may belong to bacteria of a common microflora that are noncultivable in marine agar. We conclude that most bacterial strains present in oysters are not cultivable in marine agar.

KEY WORDS: microflora, noncultivable bacteria, oyster, intergenic spacer

INTRODUCTION

Animals harbor large, active, and complex communities of microbes. Among them is the normal microflora, a population of microbes that despite its continual contact with different tissues causes no harm to the host. On the contrary, the normal microflora may have beneficial effects. It may participate in the host nutrition by complementing the process of food digestion or by contributing to the metabolism of endogenous and exogenous compounds. Normal microflora may also play a role in pathogenesis by preventing the host colonization by pathogens, controlling their growth, and modulating the immune response of the host. The composition of each particular microflora is probably determined by host properties, such as its contact history with colonizing bacteria and the dietary and environmental conditions that prevail in its habitat (Kirjavainen & Gibson 1999, Savage 1986).

At harvest oysters normally contain 10^3 to 10^5 CFU/g (De Paola et al. 1990, Wright et al. 1996), which may consist of either normal microflora or bacteria present in recently ingested food (Cook 1991, Vanderzant & Thompson 1973) or both. Bacterial flora in oysters has been described by phenotypic characterization of the strains isolated by culture, usually in marine agar (Colwell & Liston 1960, Murchelano & Brown 1968). *Vibrio* and *Pseudomonas* spp. are the bacterial species most frequently isolated from oysters. They account for 20–30% of the total CFU found in these bivalves (Colwell & Liston 1960, Murchelano & Brown 1968, Kueh & Chan 1985). The load of vibrios in the oyster's normal microflora varies according to several factors including the environmental temperature (Kaspar & Tamplin 1993) and the oyster health status (e.g., the presence of parasites) (Tall et al. 1999). Among the vibrios, important human pathogens such as *V. vulnificus* and *V. parahaemolyticus* are occasionally found in oysters (Motes et al. 1998, Kaspar & Tamplin 1993, De Paola et al. 1990, Cook 1994, Wright et al. 1996). However, since the only bacterial microflora thus far characterized in oysters is that able to grow in conventional culture media, it is likely that important strains unable to grow in such media have been missed by this kind of analysis. This situation has been described in many different bacterial habitats (Amman et al. 1995, Ueda et al. 1999, Suau et al. 1999). An example of a noncultivable bacteria found in oysters is *Cristispira pectinea*, a large spirochete that is commonly identified by its shape and large size in the crystalline style (Paster et al. 1996, Margulis et al. 1991).

The availability of methods for the detection and genetic characterization of bacteria irrespective of the microorganism's ability to grow in culture media offers the opportunity to better characterize the bacterial microflora of different animal species including the oyster (Amman et al. 1995). Two of the most commonly used methods for genetic characterization of bacterial communities are the analysis of the nucleotide sequence of 16S rDNAs (Schmidt et al. 1991) and the study of the size of the spacers found between the 16S and 23S rRNA genes (Gurtler & Stanisich 1996, Jensen & Straus 1993). This last method, though less informative, is easier to perform. The size pattern of the bacterial spacers in the community is obtained by PCR amplification of DNA extracted directly from the sample and subsequent gel electrophoresis. The pattern obtained allows for comparisons and observation of changes in the bacterial community composition. On the other hand, the nucleotide sequences of specific 16S rDNAs can be easily compared by the heteroduplex mobility assay (HMA). This assay is based on the fact that the heteroduplexes with unpaired single-stranded regions (formed as a result of the annealing of not fully complementary nucleic acid strands) display a decreased electrophoretic mobility relative to that of homoduplexes formed between identical DNA strands (Espejo et al. 1998).

Increased knowledge of the bacterial ecology of oyster microflora would greatly help the understanding of the role of bacterial microflora in digestion, metabolism, and protection from pathogens. It would also help to improve oyster management at hatcheries to avoid their colonization by pathogens to both oysters and humans. As an initial step toward the understanding of the oyster bacterial ecology, we describe here the bacterial community found in oysters according to the molecular analysis of their DNA. Results obtained from bacteria cultured in marine agar as well as those derived from bacteria directly obtained from the oysters are described.

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MATERIALS AND METHODS

Oyster Homogenate Preparation

Live oysters (*Tiostrea chilensis*) from the Quinua hatchery, Calbuco, Chile were used in our studies. Oysters in good condition, with tightly closed shells, were processed 6 h after harvest. The oysters were shucked, weighed, and an equal amount of cold sterile TEx10 (0.1 M Tris, 0.01 M ethylenediamine-tetraacetic acid) and 0.15 M NaCl (pH 7.8) was added. The oysters were subsequently homogenized in an ice bath with a Tissue Tearor (TM, model 985-370, Biospec Products Inc., WI, USA) at maximum speed for 3 min.

Culture Methods

To obtain the total cultivable bacterial count, oyster homogenates were serially diluted in 1% NaCl and plated onto marine agar. Plates were incubated for three days at 17°C (Collins et al. 1991). Putative human pathogens of the genera vibrio were detected with an enrichment protocol with alkaline peptone water (APW). Homogenate samples diluted 1:10 in APW (0.5 mL of sample in 4.5 mL of APW) were incubated without shaking for 16 h at 37°C. After enrichment, samples were plated on thiosulfate citrate bile salt sucrose (TCBS) agar and incubated for 48 h at 37°C (Wright et al. 1996).

Total Bacterial Count

Diluted homogenates were dually stained with a combination of DAPI (final concentration 5 μg/mL) and AO (final concentration 1 mg/mL) for counterstaining as described by Kuwae and Hosokawa (1999). Bacterial cells were observed using epifluorescence microscopy.

DNA Extraction from Oysters

After testing different procedures, the following DNA extraction method was adapted. The oyster homogenates were diluted (8% w/v) in TEx10/0.15 M NaCl and sodium dodecyl sulphate (SDS) was added to a final concentration of 1%. The mixture was heated at 70°C for 20 min, vigorously vortexed, and then centrifuged at 10,000 × *g* for 5 min. The supernatant was first extracted with phenol and then with a mixture phenol:chloroform (1:1). After centrifugation, the DNA in the aqueous phase was precipitated with ethanol, resuspended in sterile distilled water, and treated with RNAse A (100 ug/mL). Solid ammonium acetate (ca. 0.1 g/200 μL) was then added for a final concentration of 3 M. The samples were vortexed and centrifuged at 16,000 × *g* for 15 min, and then the DNA in the supernatant was extracted with an equal volume of chloroform:isoamyl alcohol (24:1). The DNA in the aqueous phase obtained after centrifugation was precipitated with 0.6 volumes of ice-cold isopropanol, washed once with 70% ice-cold ethanol, dried under vacuum, and then resuspended in sterile distilled water. DNA was further purified by incubation with Chelex (Bio Rad) 1% at 56°C for 30 min and then boiled for 5 min. Dilutions of supernatant were used as template for amplification.

DNA Extraction from Colonies

Marine agar plates containing 30–50 colonies were washed twice with 5 mL of cold sterile TEx10/0.15 M NaCl. The suspension was lysed with 1% SDS and DNA was extracted as described above.

Amplification Using a Single Colony as Template

Isolated strains were streaked onto marine agar and single colonies were resuspended in TE pH 8.0. Suspensions were boiled for 30 min and then centrifuged at 5,000 × *g* for 15 sec. Dilutions of the supernatants were subsequently used as templates for amplification.

PCR Amplification of 16S rRNA Genes and 16S–23S rRNA Spacers and HMA Analysis

16S rRNA genes were amplified by PCR and analyzed by HMA as described by Espejo et al. (1998). In brief, 16S rDNA in different combinations were denatured and reannealed and the electrophoretic mobility of the homoduplex and the heteroduplex was determined by polyacrylamide gel electrophoresis. The 16S–23S rDNA spacer regions were amplified by PCR and analyzed by size as described by Pizarro et al. (1996).

RESULTS

Cultivable Bacteria

Bacteria present in oysters were initially analyzed after cultivation. The oysters were obtained from a hatchery located at 40° latitude south, where the water temperature oscillates between 12°C and 14°C year-round. Two homogenates of six oysters each, harvested in August and October, rendered 10^3 and 10^5 CFU/g, respectively, when plated on marine agar. No putative pathogenic vibrio strains were observed when plated in TCBS incubated at 37°C. The genetic diversity among the bacterial colonies grown in marine agar was assessed by comparison of the sizes of their intergenic spacer regions located between the 16S and 23S rRNA genes. Of the 17 colonies obtained on marine agar (October sample), 80% were grouped into three clusters, designated A, B, and C according to the overall band pattern and the presence of common bands (Fig. 1). Group A had eight strains and was characterized by the presence of two bands of about 500 and 850 base pairs (bp), respectively. Group B had four strains and was characterized by the presence of an intense band of approximately 400 bp. Group C, in turn, had four strains and presented a band pair of about 450 and 470 bp. Pattern similarity and the number of bands in each group were variable. Group A was the most homogeneous

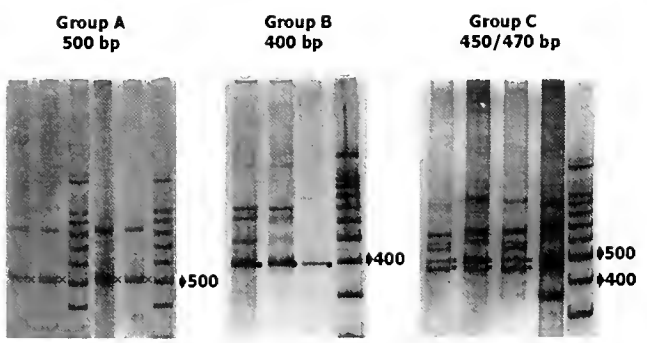

Figure 1. Polyacrylamide gel electrophoresis of the 16S–23S spacers obtained by PCR amplification of DNA from single colonies. Colonies are grouped according to the pattern of the observed spacers. X indicates the main spacer 500 bp observed in group A, ● indicates the main spacer 400 bp in group B, and * indicates the main spacer pair 450/470 bp in group C.

and group C the least. In addition, group C yielded the largest number of bands after amplification.

Cultivated strains assigned to the different groups according to their 16S–23S rDNA spacers were compared within and between groups by HMA analysis. The sequence similarity of the 16S rDNAs obtained from the different colonies following amplification was estimated by the migration of the heteroduplexes formed after denaturation and reannealing of different combination of samples. Analysis of strains belonging to groups A and B showed that within each group heteroduplexes with a slightly retarded migration were formed (Fig. 2A, lanes A1/A2 and B1/B2). This indicates that the 16S rDNAs of the bacterial strains within each of these groups were practically identical. On the other hand, hybridization between strains from groups A and B resulted in the formation of heteroduplexes with a significantly retarded migration (Fig. 2A, lane B1/A2). According to previous results (Espejo et al. 1998), this retarded migration would correspond to a nucleotide sequence difference of 15–20%. Amplification of 16S rRNA of every strain from group C rendered two products with different migration patterns (Fig. 2B, lanes C1/- and -/C2) indicating the presence of heteroduplexes. The heteroduplex nature of the slower migrating band observed in these cases was demonstrated by its disappearance after a single round of amplification following dilution (results not shown). As previously shown, dilution prevents annealing of the amplification products during the annealing step of the amplification cycle and hence formation of heteroduplexes (Espejo et al. 1998). The finding that 16S rDNAs with different sequences were obtained from a single colony was somewhat surprising but not completely unexpected. While a single colony is normally expected to contain only 16S rDNA genes with the same sequence, examples of intracellular heterogeneity of 16S rDNA are increasing (Ueda et al. 1999). On the other hand, analysis of the mobility of heteroduplexes formed between strains of bacteria of group C revealed differences in sequence similar to those observed upon amplification of a single strain (lane C1/C2). This indicates that the sequence differences of the 16S rDNAs within a single colony are comparable to the differences found between the 16S rDNAs of different bacterial strains of this group. Instead, the heteroduplexes formed between strains of group C and strains of either group A or B displayed a much slower migration compatible with a sequence difference of about 15% among these groups (lanes A1/C2, B1/C2).

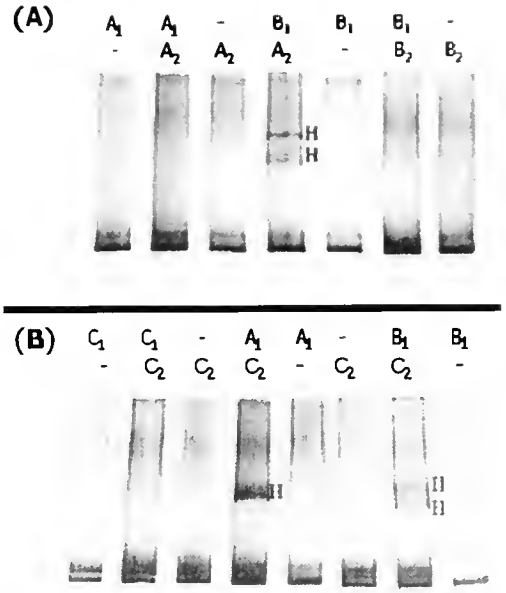

Figure 2. Sequence similarity between 16S rDNAs obtained from colonies with different 16S–23S spacer pattern estimated by HMA. The letters on the top of each lane indicate the group of the colony from which the 16S rDNA was amplified. The number identifies the colony. - indicates absence of a second 16S rDNA. H next to some bands indicates the heteroduplexes.

Noncultivable Bacteria

The bacteria characterized above seem to correspond to a small fraction of the total bacteria present in the oyster. Microscopic observations after DAPI/AO staining of the homogenates obtained from a pool of six oysters harvested in October indicated the presence of about 10^{10} bacterial cells per gram, 10^5 times more than those observed by plating. A comparison was made between the composition of the total bacteria and that of the bacteria obtained after cultivation, through the comparison of the spacer patterns observed after amplification. DNA extracted from both oyster pools and bacterial cultures was amplified and spacers resolved by polyacrylamide gel electrophoresis (Fig. 3). The patterns obtained from each preparation were very different, suggesting that the bacterial community cultured in marine agar is very different from the total bacterial population present in the oysters. The main bands observed in the amplification product of the DNA extracted from the oysters were not observed in the amplification product of the DNA extracted from the mixture of colonies recovered from the marine agar culture plates. The absence of these bands suggests, in agreement with the previous observation, that very few of the most abundant bacteria present in the oysters were able to form colonies in marine agar.

16S–23S rDNA Spacer Pattern in Individual Oysters

Since very few of the bacteria present in the oysters were able to grow in marine agar, we decided to analyze the composition of

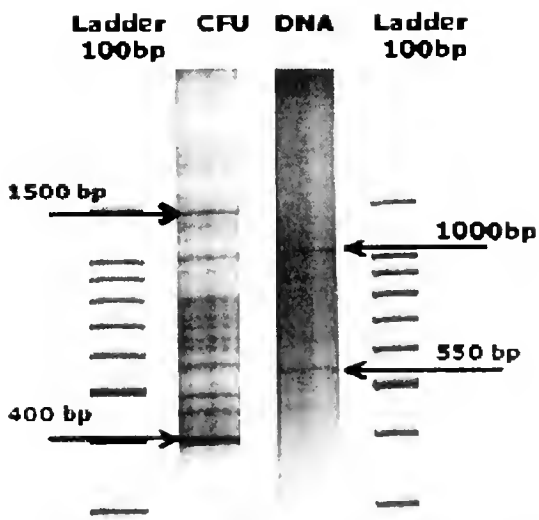

Figure 3. 16S–23S rDNA spacer pattern observed after PCR amplification of DNA extracted from the colonies grown in marine agar plates (CFU) and directly from the oyster pool (DNA). Arrows indicate the position and size of the main spacers observed in each sample.

bacteria of individual oysters by amplification of DNA extracted directly from the oysters without prior cultivation. The premise behind the idea of analyzing individual oysters was that those strains belonging to the normal microflora should be present in most of the oysters. DNA was extracted from six individual oysters and the patterns of the 16S–23S spacers, obtained after amplification, were compared. Despite the fact that different individuals had different spacer patterns, some spacers with similar migrations were present in most of the oysters. Three specific spacers were readily observable in four of the six oysters, though in different proportions (Fig. 4A). Their approximate sizes were 1000, 550, and 450 bp. In addition, the 550- and 450-bp spacers were present in six and five of the oysters, respectively. Furthermore, the 1,000- and 550-bp spacers were also detected in pooled samples of DNA obtained from the homogenates prepared from oysters harvested both in October and August (Fig. 4B).

DISCUSSION

Increased knowledge on the bacterial ecology of the oyster microflora would greatly contribute to the understanding of the role of bacterial microflora on the host digestion, metabolism, and protection from pathogens. This knowledge may be applied to hatchery management and also to postharvest processes to improve oyster quality and safety. A first requisite to ecological studies is a comprehensive knowledge of the community composition. Our results show that, as it has been observed in many other marine habitats (Amman et al. 1995), the bacteria obtained after cultivation represent only a minor fraction (<0.001%) of the total bacterial population present in the oysters. This recovery is one of the lowest reported in marine habitats (Amman et al. 1995, Schmidt et al. 1991). While the reason for this low recovery is not known, it is possible that a large part of the total bacterial count included nonviable microorganisms such as ingested allochthonous bacteria inactivated by the oyster digestion process. On the other hand, the absence of cultivable putative pathogenic vibrios, those able to grow at 37°C, may be due to the low coastal water temperature in this region (12–14°C year-round) as it is known that the presence of cultivable vibrio is greatly diminished at low temperature (Kaspar & Tamplin 1993, Motes et al. 1998). We cannot disregard, however, their presence in a viable noncultivable state as it has been described in some pathogenic *Vibrio* spp. like *Vibrio vulnificus* (Oliver 1995) and *Vibrio parahaemolyticus* (Jiang & Chai 1996).

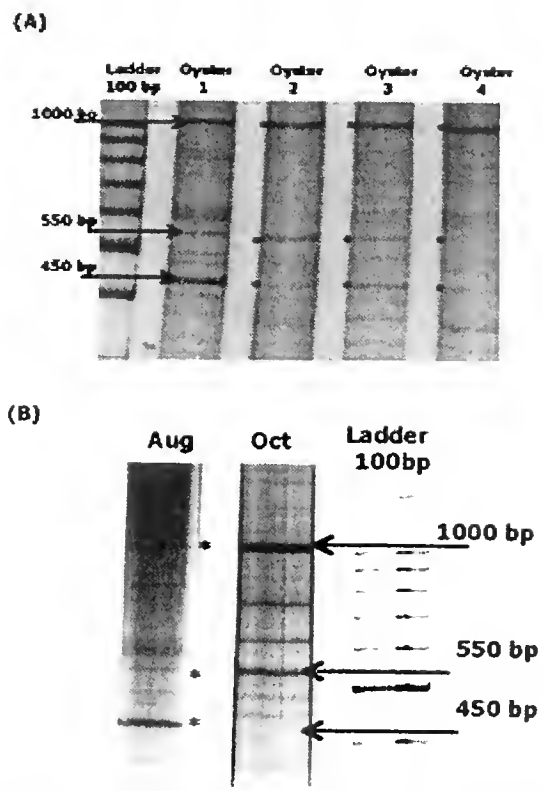

Figure 4. 16S–23S rDNA spacer observed after amplification of DNA extracted from individual oysters (A) and from pools of six oysters harvested in August and October 1999 (B). The results of only four oysters are shown (lanes oysters 1–4). Arrows and asterisks indicate the position and size of three spacers observed in most of the individual examined.

Cultivable bacterial strains may not only represent a minute fraction of the total number but also of the diversity of the microorganisms present in these bivalves. Detailed phenotypic or genotypic analysis based exclusively on the study of the bacterial community obtained after culturing seems to be of little value for a general understanding of the bacterial ecology in oysters. This does not imply that the results obtained from studies using cultivated bacteria are worthless. This type of study does give information about bacteria actually present in the hosts of study. Its limitation, however, is one of lack of generalizability. A more complete understanding can certainly be obtained from studies that combine both the analysis of cultivated bacteria as well as that of bacteria directly obtained from the host without prior cultivation.

Bearing in mind the limitation of culture studies, our results with cultured bacteria provide interesting information regarding the genetic features of these strains. The fact that most of the cultured bacteria could be grouped in three clusters according to the overall band pattern and the presence of common bands indicates that these three groups may prevail among the cultured strains. The high sequence similarity of the 16S rDNAs within the same spacer group and the dissimilarity observed between groups suggests that spacer patterns may be a useful tool to distinguish species in the oyster ecosystems. Interestingly, strains in group C showed the largest intragroup heterogeneity in spacer patterns and rendered more than one band after amplification of their 16S rDNA. As previously shown, these bands correspond to heteroduplexes formed during the last cycle of amplification by annealing between single strands of different sequences (Espejo et al. 1998). These heteroduplexes are probably due to the presence of 16S rDNAs differing in sequence. Our results add to the increasing number of reports of intracellular heterogeneity in 16S rDNA (Ueda et al. 1999).

According to the results obtained with cultured strains, the observation of particular spacers may be used as indication of the possible presence of specific bacterial strains. Considering that cultured bacteria correspond to such a low fraction of the total bacteria present in oysters, the large differences in the spacer patterns observed between bacterial DNA extracted from oysters and DNA obtained from cultured bacteria is not surprising. This large difference suggests that the groups of microorganisms observed after cultivation in marine agar do not represent the prevailing bacteria of oysters.

The spacer patterns obtained from individual oysters indicate the presence of a complex and different community of microorganisms in each individual. However, the spacers common to most

of the oysters analyzed (corresponding to approximate sizes of 1,000, 550, and 450 bp) may correspond to bacterial strains that constitute part of the normal oyster microflora and as such are present in most individuals. The apparent presence of these same spacers in pools of oysters harvested at different times of the year also supports this hypothesis. These spacers would correspond to bacteria unable to grow in marine agar because they are not observed after amplification of the DNA obtained from the cultured bacteria.

These results are similar to those obtained on the human intestinal microflora, one of the most extensively studied microbial habitats. Intestines contain 10^{10} to 10^{11} CFU/g with an overall composition that differs among individuals but that may be stable for months in the same person (Zoetendal et al. 1998). Some bacterial species appear to be present in most individuals and are considered part of the normal microflora (Zoetendal et al. 1998, Moore & Moore 1995, Matsuki et al. 1999). Many of these species can only be detected through direct DNA analysis because it has not been possible to culture them (Suau et al. 1999). In the abovementioned studies, however, the fraction of cultured bacteria is much larger than in our study.

The variations in the composition of normal microflora between individuals due to environmental and dietary differences are likely to be less pronounced in oysters than in human beings. Oysters grow in a much more homogeneous environment, which makes it more likely that they share the same diet and consequently similar microflora.

Our results suggest that in oysters there are some bacterial strains that may form part of the normal microflora. These strains, however, are not observed when the bacteria are cultured in marine agar, as is commonly the case. For an appropriate and comprehensive description of the microflora, needed for understanding the bacterial ecology in oysters, it is necessary to distinguish between bacteria of the normal microflora and allocthonous bacteria, such as opportunistic bacteria or those ingested with food. We plan to accomplish this by examination of the prevailing spacers after depuration of the oysters to remove opportunists and ingested bacteria. Once the putative normal microflora has been defined, cultivation of the bacteria containing the main spacers will be attempted using specially designed media and different culture conditions. Successful cultivation will be necessary to accomplish the phenotypic identification of the bacteria constituting the normal microflora of oysters.

ACKNOWLEDGMENTS

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ENTERIC VIRUS CONTAMINATION OF SHELLFISH: INTERVENTION STRATEGIES

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ABSTRACT Enteric viruses, such as hepatitis A, Norwalk-like caliciviruses, rotaviruses, and astroviruses, are responsible for outbreaks of food-borne illness. There are an estimated 9.2 million cases of food-borne Norwalk-like illness in the United States each year. The portion of those cases associated with shellfish is uncertain; however, shellfish are a major vector of human caliciviruses. In addition to the classical viral illnesses transmitted by shellfish, hepatitis E may become a potential threat to the shellfish consumer, particularly in Asian countries. Intervention strategies to enhance product safety include increased industry and consumer education; changes in harvesting and water monitoring practices, product management, and processing technologies; immunizations; and the development of improved detection methods.

KEY WORDS: enteric virus, hepatitis, Norwalk, rotavirus, astrovirus, illness, shellfish, intervention

INTRODUCTION

Enteric viruses encompass a broad spectrum of pathogens transmitted to food and water by the fecal-to-oral route. They include hepatitis A and E viruses, Norwalk and related caliciviruses, rotavirus, astrovirus, enteric adenovirus, and coronavirus. Molluscan shellfish are filter feeders capable of bioconcentrating viruses that are present in seawater within their edible tissues. Data on the incidence of enteric virus illness in the United States were compiled by the Centers for Disease Control and Prevention and indicate that 23 million people in the United States contract Norwalk-like viral illnesses each year, 40% of which are food-borne (Mead et al. 1999). In addition, 3.9 million people develop rotavirus and astrovirus infections, and 83,000 people develop hepatitis A (Mead et al. 1999). Only 1% of the astrovirus and rotavirus infections and 5% of the hepatitis A cases are estimated to be from foods (Mead et al. 1999). The number of illnesses caused by the ingestion of molluscan shellfish is uncertain, but shellfish are likely to be a major contributing factor for Norwalk virus illness and perhaps for hepatitis A as well. Outbreaks of hepatitis A and Norwalk-like illnesses have been linked to shellfish consumption (Richards 1988, Halliday et al. 1991). A major outbreak that was associated with the consumption of clams obtained from a mud flat outside of Shanghai, China, occurred in 1988, causing over 292,000 cases of hepatitis A over a three-month period (Halliday et al. 1991). Norwalk virus caused close to 1,500 oyster- and clam-associated illnesses in New York and New Jersey in 1982 (Richards 1985).

To date, only sporadic cases of hepatitis E have been identified; however, hepatitis E is a major cause of morbidity and mortality in Asia and may eventually spread throughout the world. In the general population, hepatitis E has a mortality rate of 0.5–3% of the individuals infected; however, the death rate is substantially higher (15–25%) in pregnant women (Mast & Krawczynski 1996).

CHARACTERISTICS OF ENTERIC VIRUSES

Human enteric viruses are only known to replicate in human and some primate cells; there is no replication in shellfish. Therefore, there is no virus amplification within foods and temperature abuse does not increase virus numbers. Within the environment, viruses may persist for weeks or months and once in shellfish may last for several weeks (Gerba & Goyal 1978). They may be preserved by cold temperatures and may be infectious in very low numbers. As few as 10–100 virus particles may be sufficient to elicit an infection (Cliver 1997).

SOURCES OF CONTAMINATION

There are numerous routes by which shellfish may become contaminated. In many cases, shellfish are impacted before harvest by contamination of the shellfish growing waters by runoff, boat waste discharge, or ineffective sewage treatment plants or septic tanks. At-harvest contamination has also been observed. Two outbreaks of Norwalk virus illness were associated with shellfish contaminated by ill harvesters who vomited overboard during harvesting operations (Kohn et al. 1995, McDonnell et al. 1997). The lack of common sense and training in good sanitation and hygienic practices are major stumbling blocks to enhancing shellfish safety. Postharvest contamination of shellfish and other foods may occur from the unsanitized hands of infected food handlers, contaminated processing surfaces, cross-contamination of the cooked with raw product, or by postharvest adulteration. Shellfish may be adulterated postharvest through the use of unsanitary water, employed either as a food additive or for rinsing product or processing equipment; ice or food additives containing viral contamination; packaging materials; or unsanitary transport or storage conditions. Groups at risk of criminal or civil penalties in the event of a food-borne outbreak include shellfish growers, harvesters, and processors; transporters; retail and wholesale outlets; restaurants; and government regulatory agencies. One prominent outbreak of hepatitis A occurred from the consumption of contaminated oysters from Wallis Lake in New South Wales, Australia (Conaty et al. 2000) and led to a class action suit. The courts found the industry and regulatory agencies liable for millions of dollars in damages because they did not exercise adequate measures to prevent outbreaks of hepatitis A.

STRATEGIES TO REDUCE ENTERIC VIRUSES IN SHELLFISH

Strategies to reduce the incidence of shellfish-related illness must incorporate preharvest, at-harvest, and postharvest interventions (reviewed by Richards, 2001). Some practical approaches directed toward the reduction of preharvest contamination are: (1) improved operation and monitoring of sewage treatment plants that impact coastal areas, (2) elimination of improperly functioning septic tanks from coastal areas, (3) better enforcement of shellfish

harvesting and tagging regulations, (4) enhanced enforcement of ocean-dumping restrictions, and (5) improved shellfish monitoring. Shellfish tagging should be monitored to ensure that shellfish are only obtained from areas approved for harvesting. At-harvest contamination may be reduced by preventing sick shellfishermen from harvesting or handling shellstock. In addition, the discharge of boat wastes, particularly feces, vomitus, and urine, should be strictly forbidden. Postharvest strategies to reduce illness should involve various types of processing to reduce or eliminate potential viral contaminants. Processing may include: (1) thorough washing of shellstock; (2) heating/cooking of shellfish, either in-shell or shucked; (3) irradiation or dehydration of product; and/or (4) depuration and relaying. Thorough cooking is effective in eliminating enteric viruses, but the shellfish may lose many of their desirable characteristics if they are fully cooked. They may become tough, dry, and lose their flavor. In addition, high protein and lipid levels, as are found in shellfish tissues, tend to protect the viruses from thermal inactivation (Filippi & Banwart 1974, Bidawid et al. 2000).

Irradiation can effectively eliminate enteric viruses from shellfish. Ionizing radiation, microwave energy, and ultraviolet irradiation are effective in reducing virus levels. Ionizing radiation was effective in substantially reducing enteric viruses in shellfish, but required a dose that caused rapid shellfish mortalities (Mallett et al. 1991). The use of ionizing radiation may be limited to the disinfection of products destined for shucking. Microwave energy is effective in eliminating shellfish pathogens by virtue of cooking. Uneven heating and the problems associated with marketing a fully cooked shellfish product reduce the feasibility of microwave processing for shellfish. Ultraviolet light can inactivate only surface contaminants, but may be applied in a depuration setting to inactivate pathogens purged from the shellfish during the depuration process. Depuration is the commercial process where shellfish are placed in tanks of clean seawater and allowed to purge contaminants for a short period of time. Ultraviolet light is often used to disinfect the depuration water as it is recirculated through the tank. Over a two- to three-day processing cycle, molluscan shellfish will expel many of the bacteria and viruses, and much of the sediment from their digestive diverticula. Unfortunately, shellfish are unable to purge all the enteric viruses. Some viruses migrate from the digestive diverticula into the epithelial cells surrounding the lumen of the gut and become sequestered within the shellfish tissues (Hay & Scotti 1986, Richards 1990). Since only a few viruses may be sufficient to cause infection, these entrapped viruses limit the effectiveness of depuration in producing a safe product.

Relaying is where shellfish are removed from contaminated shellfish growing areas and placed in clean shellfish growing waters for an extended period (weeks to months) (Richards 1988). Through natural virus inactivation processes, relaying offers some advantages in eliminating viruses that might be sequestered within the shellfish tissues. However, relaying is only effective if the waters remain clean during the entire relay process. This is often difficult to establish since heavy rains, malfunctioning treatment plants, or illegal boat waste discharge could occur at any time during the extended relay period. In addition, shellfish harvesters, pressured to meet customer demands, may be tempted to prematurely harvest shellfish.

Intervention strategies may also include immunizations of food processors and handlers, the general population, children, or just high-risk individuals. There is no consensus on who should be immunized, and the costs and logistics of immunization programs can be daunting. Today, there are vaccines for hepatitis A virus, but the vaccine for rotavirus illness was taken off the market to await further testing. There is no vaccine currently for Norwalk-like viruses; however, vaccines are emerging for hepatitis E virus, which may become a serious public health concern in many countries over the next decade. Immunization programs for food handlers would be extremely difficult to monitor since much of the workforce consists of teenagers who quite frequently move from job to job.

The shellfish industry is increasingly subscribing to the provisions of the hazard analysis critical control point (HACCP) program. The key to HACCP is the delineation of the critical control points in the passage of product from the ocean to the consumer. Under HACCP, the critical control points are monitored to ensure the points are maintained within acceptable limits. Monitoring is practical for some aspects of shellfish sanitation and processing; however, methods to monitor viral contaminants are limited. Consequently, HACCP falls short of protecting the consumer from enteric viruses.

Virus extraction and analytical methods have been developed for a variety of enteric viruses in shellfish. Over the past decade, most of the procedures developed have been based on molecular biological methods, principally reverse transcription-polymerase chain reaction (RT-PCR). Unfortunately, molecular biologically based tests do not differentiate between infectious and inactivated viruses and are only marginally effective in the evaluation of shellfish (Richards 1999). More conventional cell culture-based assays are capable of detecting infectious viruses, but only if the viruses are capable of being propagated in cell or tissue culture. To date, no one has successfully propagated Norwalk-like viruses *in vitro*, and most of the wild-type hepatitis A viruses are incapable of being propagated *in vitro*. Consequently, further research is needed to identify better methods to monitor for the presence of infectious viruses in shellfish.

Overall, there are several measures that can be employed to increase the virological safety of shellfish. The regulation and monitoring of harvesting areas by State and Federal agencies are crucial in providing reasonably clean shellfish in the marketplace. Shellfish must be obtained only from shellfish beds approved for harvesting based on sound sanitary criteria. Proper tagging of raw products will facilitate tracing back to the original source in the event of an outbreak, thus allowing contaminated products to be taken off the market. Efforts to educate shellfish harvesters, processors, and handlers should be enhanced to stress the importance of following the regulations. Thorough cooking is the most effective and simplest method to inactivate enteric viruses in shellfish. If a raw product is desirable, extended relay of moderately or lightly contaminated shellfish into clean water is the most practical procedure to decontaminate large numbers of shellfish. Remediation of shellfish growing waters may be accomplished by continuously monitoring and improving sewage treatment plants and septic systems and by strict enforcement of laws against boat waste discharge. New molecular biological- and cell culture- based assays for the detection of enteric viruses are under development. Future efforts to avert outbreaks of enteric virus illness will likely rely on a combination of enhanced enforcement of existing laws, improved processing and handling techniques, and direct virus testing.

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DETECTION AND CHARACTERIZATION OF TOXIGENIC BACTERIA ASSOCIATED WITH *ALEXANDRIUM CATENELLA* AND *AULACOMYA ATER* CONTAMINATED WITH PSP

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ABSTRACT The paralytic shellfish toxins (PST) are potent neurotoxins. Among them, saxitoxin (STX) is one of the most dangerous for humans and animals, functioning by blocking sodium channels in mammalian nerve cells. Marine algae, such as the dinoflagellate *Alexandrium catenella*, are generally believed to be responsible for the production of STX, and if PSTs are produced by the plankton, they can be concentrated by filter-feeding organisms, effectively increasing the risk of poisoning for consumers of shellfish. However, it is not clear whether *A. catenella* itself, or the bacteria associated with it, produce the toxins and, furthermore, whether toxigenic bacteria are associated, as well, with shellfish contaminated with PST. Bacteria able to produce sodium channel blocking (SCB) toxins were isolated from a toxin-producing *A. catenella* culture and from shellfish (*Aulacomya ater*) contaminated with PST from the Magallanes region of southern Chile. Quantitative levels of PST and bacterial SCB toxins were ascertained by mouse assay and mouse neuroblastoma assay, respectively. More than 60% of the bacteria enriched and analyzed from *Aulacomya ater* and *A. catenella* were observed to produce detectable amounts of SCB. The SCB toxin-producing bacterial isolates were characterized by amplified 16S ribosomal DNA restriction analysis (ARDRA), and selected isolates, representing the different ARDRA groups, were identified by 16S rDNA sequence comparisons and estimations of their phylogenetic relationships with validly published species. Most of the SCB toxin-producing isolates from *A. ater* and from *A. catenella* represent new species of genera within the *Proteobacteria* and, in some cases, demonstrate close phylogenetic relationships with previously reported toxigenic bacteria isolated from other dinoflagellate cultures. However, the isolates from *A. ater* represent different taxonomic lineages from those associated with the dinoflagellate *A. catenella*. These observations suggest that SCB toxin contamination of shellfish may occur independently of algal contamination.

KEY WORDS: toxigenic bacteria, *Alexandrium catenella*, dinoflagellate, shellfish, PSP, saxitoxin

INTRODUCTION

Paralytic shellfish toxins (PST), comprising at least 20 chemically similar derivatives (Oshima et al. 1993) including saxitoxin (STX), are potent neurotoxins that cause paralytic shellfish poisoning (PSP) in humans. PSTs act by blocking sodium channels in mammalian cells, thus preventing conductance of signals along neurons and effectively paralyzing muscle activity (Baden & Trainer 1993). Much evidence has suggested that the PSTs originate from marine dinoflagellates (e.g., of the genera *Alexandrium* and *Gymnodinium*) (Anderson et al. 1990, Cembella 1998). If toxins are present in the plankton, they are concentrated by filter-feeding shellfish and subsequently conveyed to humans via their consumption.

Along the southern Chilean coast, between 1972 and 1998, 348 cases of PSP, including 23 deaths, were reported (Suárez-Isla & Guzmán 1998). The incidences of PSP, in all cases, were associated with the consumption of PST-contaminated mussels. Monitoring also detected the dinoflagellate *Alexandrium catenella* in the PST-contaminated regions, and this species is believed to be responsible for the toxicity in the shellfish (personal communication, Dr. L. Guzmán, Instituto de Fomento Pesquero, IFOP Magallanes, Chile). Sodium channel-blocking (SCB) toxins (i.e., STX) has been detected also in cultures of several species of cyanobacteria: *Aphanizomenon flos-aquae* (Mahmood & Carmichael 1986), *Anabaena circinalis* (Humpage et al. 1994), *Lyngbya wollei* (Carmichael et al. 1997), and *Cylindrospermopsis raciborskii* (Lagos et al. 1999). Further, Negri and Jones (1995) reported high levels of PST in a freshwater mussel when fed with the neurotoxic cyanobacterium *Anabaena circinalis*. Recently, reports have described heterotrophic bacteria as well, isolated from cultures of toxic dinoflagellates, that are capable of producing SCB toxins (Kodama et al. 1988, Doucette & Trick 1995, Franca et al. 1996, Gallacher et al. 1997). These bacteria have been associated with different dinoflagellate species from different geographic regions and represent different taxonomic groups. However, few toxigenic bacteria isolated from shellfish have been described to date (Freitas et al. 1992).

To define further the diversity and potential of toxigenic bacteria associated with PST contamination of shellfish along the southern Chilean coast, isolated bacteria were enriched from mussels (*Aulacomya ater*), collected along the Magallanes coastline of southern Chile, and from a culture of a toxic dinoflagellate (*A. catenella*). The cultivable bacteria from the shellfish and from the dinoflagellate culture were analyzed for SCB toxin production, and the bacterial isolates determined to be toxigenic were characterized systematically.

METHODS

Isolation and Cultivation of Bacteria from PST-Contaminated Mussels (Aulacomya ater) *and from a Toxic Dinoflagellate Culture* (Alexandrium catenella)

Mussels (*Aulacomya ater*) were collected from the Magallanes region of the southern Chilean coast, near Seno Europa (50°03′S, 74°21′W). After collection, the mussels were maintained at 4°C until analysis. The toxicity of the shellfish, as determined by the AOAC mouse bioassay (Williams 1984), was 560 ± 200 μg PST/100 g of mussel flesh (personal communication, Dr. L. Guzman, Instituto de Fomento Pesquero, IFOP Magallanes, Chile). Mussel

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tissue was homogenized in NaCl (1%, w/v), using a Tissue Terror. Dilutions of the homogenate were then inoculated into marine broth (Difco Laboratories, Detroit, MI, USA), and 0.1 mL of each dilution was plated onto marine agar medium, prepared with 28‰ sea water. These cultures were incubated at 17°C for at least one week. One hundred colonies with different morphologies were picked and plated individually on marine agar plates, followed by incubation at 17°C for 72 h.

A SCB toxin-producing culture of *Alexandrium catenella*, derived from clone ACC07 from the Aysen region (45°32′S, 73°34′W) in southern Chile, was obtained from Dr. Myriam Sequel (Instituto de Fomento Pesquero, Puerto Montt, Chile). Dilutions of the *A. catenella* culture were prepared, and 0.1 mL were then plated on marine agar medium. These cultures were incubated at 17°C for at least one week. Twenty colonies with observable morphological differences were picked, plated, and incubated as previously described.

Analysis of Toxin Production by Bacterial Isolates

Each bacterial colony was inoculated into 30 mL of marine broth. After incubation at 23–25°C for 36 h on a rotary incubator (100 oscillations per minute), the culture was centrifuged (10,000 × *g*, 20 min), and the supernatants were collected and stored at –20°C until analyzed.

SCB activity in bacterial culture supernatants was measured using a cell-culture assay with mouse neuroblastoma (MNB) (Gallacher & Birkbeck 1992). An STX dose-response curve, using a saxitoxin standard (NRC, Halifax, Canada), was prepared for the range of 0 to 500 nM. All isolate supernatants were tested in triplicate, and controls were done in quadruplicate repeats, as described by Gallacher et al. (1997). The supernatant samples used in the assays were prepared at 1/10 dilutions for all screenings. SCB toxin levels were compared to STX dose-responses to estimate SCB activities with respect to nM STX-equivalents.

Phenotypic and Genotypic Analyses of Toxigenic Bacterial Isolates

Bacterial isolates were characterized by their colony morphology, cultivated on marine agar medium. Staining and microscopic characterization were carried out using standard microbiological methods (Murray & Robinow 1981). API 20 NE strips (BioMerieux Viteck, Inc., Hazelwood, MO, USA) were used as described in API 20 NE protocols for determination of a standard set of phenotypic characteristics. The genotypic diversity of SCB toxin-producing bacteria isolated from the mussel (*A. ater*) tissue samples and from the dinoflagellate (*A. catenella*) culture was investigated using amplified 16S ribosomal DNA restriction analysis (ARDRA) (Vaneechoutter et al. 1992). Bacterial DNA was purified from the isolates as described previously (Espejo et al. 1998). 16S rRNA genes were targeted and amplified by polymerase chain reaction (PCR) using primers and the reaction conditions described by Moore et al. (1996). Subsequently, the amplified 16S rDNA was restricted overnight with 2.5 U of *Hae*III (Gibco BRL, Eggenstein, Germany) or *Msp*I (Gibco BRL) at 37°C or with 5 U *Taq*I (Qiagen GmbH, Hilden, Germany), for three hours at 65°C. The resulting restriction fragments were separated by electrophoresis in 1.5% (w/v) agarose and detected by staining with ethidium bromide (0.5 μg mL^{-1}).

The PCR-16S rDNA was purified using Microcon Centrifugal Filter Devices (Millipore Corp. Bedford, MA, USA) in preparation for nucleic acid sequence determinations and sequenced directly using an Applied Biosystems Inc. 373A DNA Sequencer and the protocol of the manufacturer (Perkin-Elmer, Biosystems, Foster City, CA, USA) for *Taq* cycle-sequencing with fluorescent dye-labeled dideoxynucleotides. The sequencing primers for 16S rRNA have been previously described (Lane 1991).

Analysis of the sequence data and preliminary identifications of the isolates were carried out using the EBI (Baker et al. 2000) interactive Fasta3 search algorithm (Pearson & Lipman 1988).

RESULTS AND DISCUSSION

Nineteen bacterial isolates, picked on the basis of observable morphological differences, were obtained from a culture of an SCB toxin-producing strain of *Alexandrium catenella*. Eleven of these isolates were observed to produce SCB toxin at levels greater than 0.002 fg STX equiv $cell^{-1}$ (Table 1). One of these isolates, MV20, produced amounts of SCB toxins comparable to the level reported by Kodama et al. (1988) for a strain of *Moraxella* sp. All bacteria isolated from the *A. catenella* culture were rod-shaped, Gram-negative, and characterized with the phenotypes listed in Table 2. Five distinct restriction patterns were observed for the 11 toxin-producing isolates, and the 16S rDNAs from two representatives of each ARDRA type were sequenced. The sequence data obtained were compared with reference sequences in the EMBL (Baker et al. 2000), and the approximate identifications of the isolates relative to validly published species are shown in Table 3. As can be expected and was possible to observe, the strains that were closely related, on the basis of their 16S rDNA sequence similarities, also possessed similar phenotypic characteristics (Tables 2, 3). The 16S rDNA sequences determined for the bacteria isolated from the SCB toxin-producing *A. catenella* culture corresponded, in most of the cases, to new species of *Sulfitobacter*, a genus belonging to the α-subclass of the *Proteobacteria* and closely related with the *Roseobacter* phylogenetic lineage (González & Moran 1997). Recently, in separate studies, bacteria of the genus *Roseobacter* or closely related genera have been observed to be associated with toxic dinoflagellates (Lafay et al. 1995, Prokic et al. 1998). *Roseobacter* species appear to be involved in important biogeochemical functions in marine coastal regions, presumably in the cycling of organic and inorganic sulfur (González et al. 1999). These bacteria are able to utilize sulfur compounds provided by phytoplankton, macroalgae, and coastal vascular plants. In that context, it is not surprising to observe species related with the *Roseobacter* group associated with dinoflagellates. Additionally, two isolates, MV05 and MV07, were unrelated with any validly published species and probably represent new species and genera belonging to the γ-subclass of the *Proteobacteria*. The

TABLE 1.

Proportion of SCB toxin-producing bacterial isolates associated with *A. catenella* and *A. ater*.

Origin	Number of Bacterial Isolates Analyzed	Bacteria Producing SCB Toxins (%)*,**
Alexandrium catenella	19	58
Aulacomya ater	80	77

* As determined by the Mouse Neuroblastoma Assay (Gallacher & Birkbeck 1992).

** Toxicity between 20 nM until 100 nM STX eq.

TABLE 2.

Characteristic of isolates from dinoflagellates (*Alexandrium catenella*) and mussels (*Aulacomya ater*).

Characteristic	MV-3	MV-5	MV-7	MV-8	MV-11	MV-20	CH-32	CH-34	CH-61
Cell shape	short rod	short rod	short rod	short rod	short rod	short rod	short rod	short rod	short rod
Gram	−	−	−	−	−	−	−	−	−
Motility	−	+	+	−	−	−	−	−	−
Reduction nitrate to nitrites	−	+	+	+	+	+	−	−	−
Indole production	−	−	−	−	−	−	−	−	−
Glucose fermentation	−	+/−	−	−	−	−	−	−	−
Arginine dihydrolase	−	−	−	−	−	−	−	−	−
Urease	−	+	−	−	−	+	−	−	−
Hydrolysis (B-glucosidase)	−	+	+	−	−	−	−	−	−
Hydrolysis gelatine	−	−	−	−	−	−	−	−	−
Cytochrome oxidase	+	+	+	+	+	+	+	+	+
B-galactosidase	−	+	+	+	−	+	−	−	−
Growth on									
Glucose	+/−	+	+	+/−	−	+	−	−	−
Arabinose	+/−	+	+	+/−	−	+	−	−	−
Mannose	+/−	−	−	−	−	−	−	−	−
Mannitol	−	+	+	+/−	−	+	−	−	−
N-Acetyl-glucosamine	−	−	−	−	−	−	−	−	−
Maltose	−	−	−	+/−	−	−	−	−	−
Gluconate	−	−	+	−	−	−	−	−	−
Caprate	−	−	−	−	−	−	−	−	−
Adipate	+	+	+	+	−	+	−	−	−
Malate	+/−	+	+	+	+	+	+/−	−	+
Citrate	−	−	−	−	−	+	−	−	−
Phenyl-acetate	−	−	+	−	−	−	−	−	−

bacterial diversity in the cultivable community associated with the culture of the SCB toxin-producing *A. catenella* was relatively limited, although a markedly high percentage of the isolates were observed to produce SCB toxins.

One hundred isolates able to grow on marine agar medium were obtained from homogenized tissue of *Aulacomya ater* contaminated with PSP (personal communication, Dr. L. Guzman, IFOP, Magallanes, Chile). *A. ater* is a mussel found typically along the Chilean coast. Eighty of the bacterial isolates were picked for analysis, and a high percentage (77%) was observed to produce SCB in the range between 20 and 100 nM STX equivalents (Table 1). Of the isolates that tested positive for toxin production, 30 with the highest measurable levels of toxin were picked for further analyses. All bacterial isolates from *A. ater* tissue were Gram-negative rods and the phenotypic characteristics for the isolates analyzed (Table 2). On the basis of 16S rDNA sequence comparisons, the 30 isolates were observed to be closely related with species of the genera *Psychrobacter*, of the γ-Proteobacteria subdivision (Table 4). The isolates analyzed were not distinguishable by biochemical testing (Table 2), although 16S rDNA sequence similarities indicated that they comprise at least six distinct clusters, probably corresponding to different species. Although no accepted level of 16S rDNA sequence similarity exists for describing bacterial species, experience has shown that bacteria with 16S rDNA sequence dissimilarities of more than 1% almost certainly comprise different species (unpublished data). Additional phenotypic characteristics must be found to differentiate these bacteria. *Psychrobacter* species and related organisms are found typically in Antarctic sea ice and Antarctic ornithogenic soil (Bowman et al. 1996, 1997). Species of the *Psychrobacter* genus are characteristically psychrophilic or psychrotolerant, as well as halotolerant, with a distribution of species in marine and terrestrial environments. The mussels from which the isolates were obtained were collected in a coastal region with salinity ranging between 22 and

TABLE 3.

Phylogenetic/taxonomic relationships among toxic marine dinoflagellate-associated bacterial isolates.

Bacterial Isolates	Closest Phylogenetic Relative*	16S rRNA Gene Sequence Similarity (%)**	Other Relatives	Phylogenetic Taxon
MV03	*Sulfitobacter mediterraneus*	95.5	*Roseobacter* spp.	Proteobacteria α
MV05	*"Oceanospirillum" pusillum*	89.3	unidentified isolates	Proteobacteria γ
MV07	*"Oceanospirillum" pusillum*	87.4	unidentified isolates	Proteobacteria γ
MV08	*Sulfitobacter pontiacus*	94.3	*Roseobacter* spp.	Proteobacteria α
MV11	*Sulfitobacter mediterraneus*	96.4	*Roseobacter* spp.	Proteobacteria α
MV20	*Sulfitobacter pontiacus*	96.0	*Roseobacter* spp.	Proteobacteria α

* Validly published species.

** FASTA analysis (Pearson & Lipman 1988).

TABLE 4.

Phylogenetic/taxonomic relationships among toxic marine shellfish-associated bacterial isolates.

Bacterial Isolates	Closest Phylogenetic Relative*	16S rRNA Gene Sequence Similarity (%)**	Phylogenetic Taxon
CH03	*Psychrobacter glaciincola*	97.5	Proteobacteria γ
CH04	*Psychrobacter glaciincola*	95.7	Proteobacteria γ
CH08	*Psychrobacter glaciincola*	97.9	Proteobacteria γ
CH22	*Psychrobacter pacificensis*	96.2	Proteobacteria γ
CH25	*Psychrobacter pacificensis*	93.5	Proteobacteria γ
CH32	*Psychrobacter glaciincola*	98.1	Proteobacteria γ
CH34	*Psychrobacter glaciincola*	98.1	Proteobacteria γ
CH47	*Psychrobacter glaciincola*	95.7	Proteobacteria γ
CH61	*Psychrobacter glaciincola*	96.7	Proteobacteria γ
CH64	*Psychrobacter glaciincola*	98.1	Proteobacteria γ
CH71	*Psychrobacter glaciincola*	96.4	Proteobacteria γ
CH72	*Psychrobacter glaciincola*	98.1	Proteobacteria γ
CH88	*Psychrobacter glaciincola*	99.6	Proteobacteria γ

* Validly published species.

** FASTA analysis (Pearson & Lipman 1988).

26 gL^{-1} and water temperatures of 6–7°C (depths 0–10 m). It is probably not surprising, at these latitudes, to find psychrophilic or psychrotolerant bacteria associated with the shellfish. Furthermore, the low water temperature may be one reason to have found a lower diversity of cultivable bacteria associated with mussels than what one might expect in more temperate or tropical environments.

This is the first case of SCB toxin production observed in species of *Psychrobacter* and *Sulfitobacter* genera, which suggests that toxin-production in bacteria are not limited to species of *Moraxella*, *Alteromonas*, and *Pseudomonas*, as has been suggested from the results of earlier studies (Doucette & Trick 1995, Kodama et al. 1988). The high percentage of SCB toxin-producing bacteria isolated from the PST-contaminated mussels and from the dinoflagellate culture in this study, as well as results in other previous reports (Gallacher et al. 1997), suggest that this property is not uncommon among marine bacteria. However, SCB toxin is not necessarily related only with saxitoxin, and further analyses (e.g., by other methods such as HPLC and mass spectrometry) need to be included to reliably identify the toxin patterns.

Interestingly, in this study, the range of bacterial diversity obtained from the toxin-contaminated mussels and from the toxic dinoflagellate culture were relatively limited, although past studies using enrichment cultivation methods (Kodama et al. 1988, Doucette & Trick 1995) also recovered only a limited range of bacterial species associated with marine algae. Questions about whether specific species or communities of bacteria are associated with certain algal species or with algal species in defined environmental situations have never been addressed to any degree. Furthermore, the observed high percentage of toxigenic isolates among the cultivable bacteria from the mussels and the dinoflagellate culture raises interesting questions with respect to the influence the associated bacteria may have on production of toxin by the algae and accumulation of toxin by the shellfish. However, the toxin-producing bacteria obtained from the PST-contaminated mussels comprised species from different phylogenetic lineages than those isolated from the toxin-producing dinoflagellate. These observations suggest that the accumulation of PSTs in shellfish may not be dependent only upon the occurrence of toxin-producing marine algae. While it is clear that the shellfish are filtering phytoplankton, including SCB toxin-producing dinoflagellates such as *A. catenella*, the shellfish may have also been obtaining bacteria, the majority of which are found on particulate matter in the marine environment. In the case of our study, the shellfish were collected at a time when monitoring was not detecting significant numbers of *A. catenella* in the water column. However, the shellfish contained high levels of PST. Thus, although the Magallanes region was infected with *A. catenella* blooms at earlier monitoring times, there was no direct influence of marine algae on the toxin levels of the shellfish at the time of our study. It should also be noted that there is usually a lag between the marine algal bloom (in water column) and the detection of PSTs in the shellfish. Results also suggest that toxin-producing bacteria may play a much more important role in PST-contamination of shellfish than has been accepted in the past. Obviously, much more extensive sampling and analysis must be carried out for such a working hypothesis to be tested with any reliability.

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PHYTOPLANKTON COMPOSITION AND *PYRODINIUM BAHAMENSE* TOXIC BLOOMS IN MANILA BAY, PHILIPPINES

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ABSTRACT *Pyrodinium bahamense* var. *compressum* has caused approximately 1,992 paralytic shellfish poisoning (PSP) cases and 116 deaths in the Philippines and has remained the main cause of PSP in the tropical world. The first toxic bloom of the organism in Manila Bay, Philippines was in 1988. Since then the recurrence of the event (usually beginning in April/May and ending in September/October) has been experienced every year except in 1997 and 1999. The latest incident of the phenomenon was in 1998 followed by the absence of the species in 1999 during the months it was present in previous years. Eight stations in Bataan and Cavite, two red tide prone areas in Manila Bay, were sampled during three seasons from 1997 to 1999 for phytoplankton abundance and species composition. Bataan and Cavite, though situated within the same bay, have distinct differences during the three seasons with respect to dinoflagellate population. There is a pronounced increase in dinoflagellate population in Bataan from northeast monsoon to tradewinds to southwest monsoon. In Cavite, dinoflagellates constitute a minor portion of the phytoplankton community during the whole year. In 1998, *Pyrodinium* blooms in both areas started in April/May and terminated in October. A bloom of the heterotrophic dinoflagellate *Noctiluca scintillans* in Bataan and the dominance of the diatom *Chaetoceros* spp. in Cavite succeeded the *Pyrodinium* bloom. *N. scintillans* was observed to be present in the two sites during the entire duration of the study.

KEY WORDS: *Pyrodinium bahamense*, paralytic shellfish poisoning, toxic blooms, Manila Bay

INTRODUCTION

Toxic red tides are recurrent events in the Philippines and have been caused so far only by one dinoflagellate species, *Pyrodinium bahamense* var. *compressum*. Manila Bay is one of the 16 bays affected by this Paralytic Shellfish Poisoning causative organism.

Analysis of the phytoplankton population is an important tool in the proper management of toxic red tides in the country. This helps in the identification of *Pyrodinium* and other potentially harmful algal species that dominate the phytoplankton population during different seasons and the conditions favoring their bloom. Bajarias (1999) has provided an inventory of the dinoflagellates present in 11 marine coastal areas in the Philippines including Manila Bay.

There has been no published work on phytoplankton distribution in Manila Bay. This paper for the first time provides the relative abundance of dinoflagellates and diatom species in two toxic red tide affected areas in the bay for the three different seasons for the period 1997 to 1999.

MATERIALS AND METHODS

Manila Bay is a semi-enclosed bay located at the western coast of Luzon Island, Philippines (lat. 14°53′N; long. 120°76′E) and bounded by the provinces of Cavite in the south, Metro Manila and Rizal in the east, Bulacan and Pampanga in the north, and Bataan in the west and northwest. The bay was first affected in 1988 and annual occurrences of toxic red tides have been recorded (Azanza 1997).

Bataan and Cavite are two areas within Manila Bay that are commonly utilized for shellfish farming and harvest from the wild. Both these areas have a history of red tide occurrences. Phytoplankton monitoring in eight stations in Bataan and Cavite (Fig. 1) was conducted three times for three years covering different seasons (northeast monsoon, tradewinds, and southwest monsoon). Climatological data was obtained from the Philippine Atmospheric, Geophysical and Astronomical Services Administration (PAGASA). Data for Bataan was obtained from Port Area, which is the weather station nearest to the study site.

Samples for phytoplankton analysis were collected using a plankton net with mesh size of 20 μm towed vertically in the water

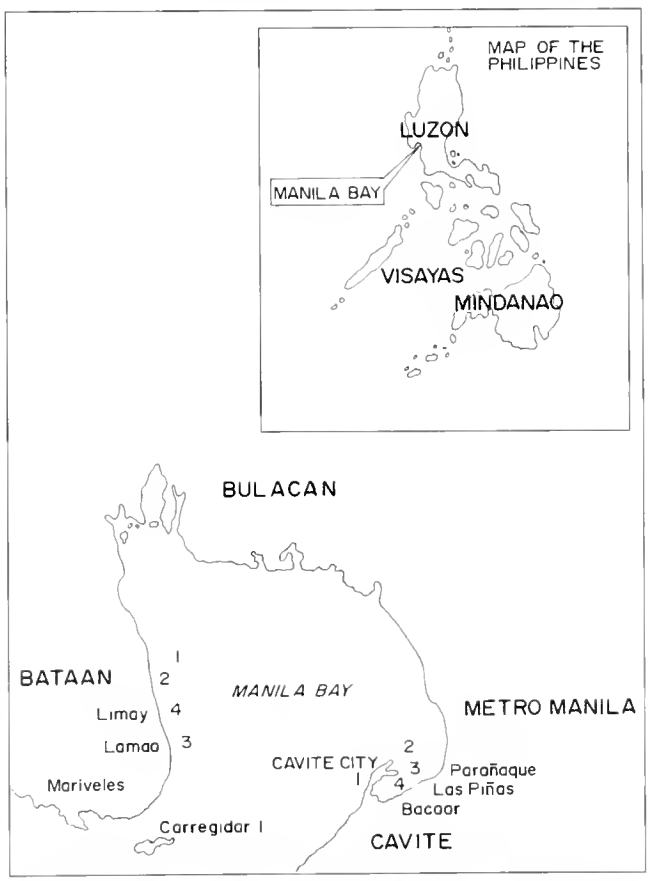

Figure 1. Map of Manila Bay showing the sampling stations in Bataan and Cavite.

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column. The cells were preserved by the addition of 1% Lugol's solution. Quantification of plankton was carried out using a Sedgewick Rafter chamber following the technique of Azanza (1997) and Corrales et al. (1995). Identification of phytoplankton was done using the taxonomic keys of Taylor (1976) and Tomas (1997).

RESULTS

Climactic Condition from 1997 to 1999

The climatological conditions during the three years of study are summarized in Figure 2. Cavite had a relatively higher monthly mean air temperature than Bataan. The highest mean air temperature recorded in both places was in the range 34–35°C, occurring during tradewinds in the months of April to May (Fig. 2a). The general rainfall conditions in 1997 and 1998 were relatively similar for the two sites. The amount of rainfall was higher during May to August of 1997, while from 1998 to 1999 there was little variation in the amount of rainfall in both sites (Fig. 2b). The wind force in Cavite ranges from 2–6 mps, while in Bataan it ranges from 2–4 mps (Fig. 2c).

Phytoplankton Composition

The phytoplankton community was composed mainly of diatoms and dinoflagellates. The diatoms were more dominant than dinoflagellates in all samples for the three seasons in the three years of study. There were 10 families of dinoflagellates, nine families of diatoms, and two marine flagellates (raphidophytes and chrysophytes) found in Manila Bay (Table 1). Under dinoflagellates the families *Gonyaulacaceae, Ceratiaceae, Dinophysiaceae, Gymnodiniaceae, Prorocentraceae, Noctilucaceae,*

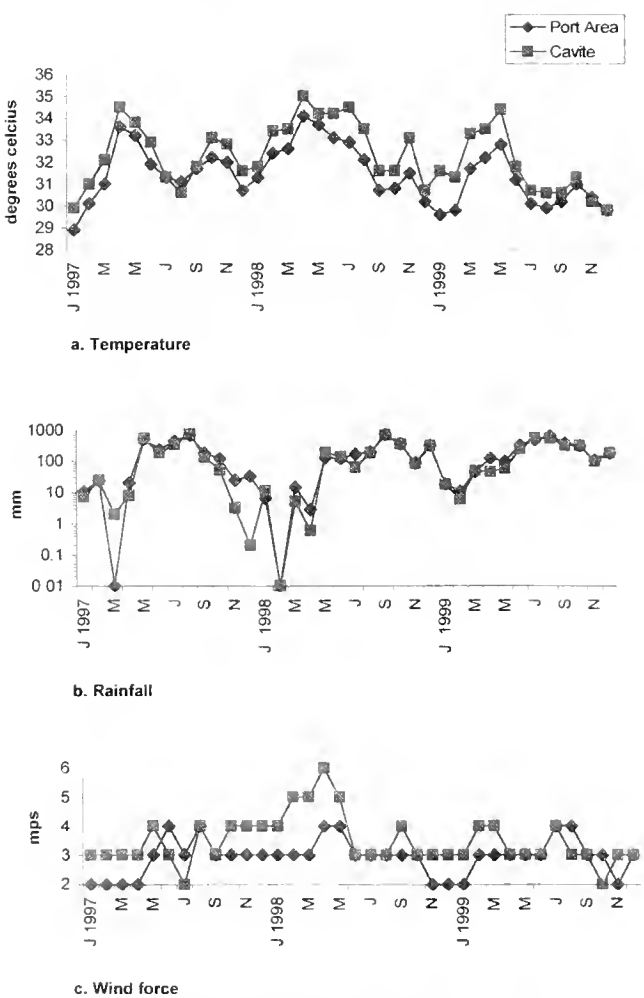

Figure 2. Climatological conditions from 1997 to 1999.

TABLE 1.

List of phytoplankton species identified in Manila Bay, Philippines.

Family	Species
Dinoflagellates	
Gonyaulacaceae	*Gonyaulax spinifera*
	Alexandrium sp.
	Pyrodinium bahamense var. *compressum*
	Goniodoma sp.
Ceratiaceae	*Ceratium furca*
	Ceratium macroceros
	Ceratium tripos
	Ceratium trichoceros
	Ceratium fusus
	Ceratium breve
	Ceratium carriense
	Ceratium vultur
Dinophysiaceae	*Dinophysis caudata*
	Dinophysis rotundata
	Dinophysis miles
Gymnodiniaceae	*Gyrodinium spirale*
	Gyrodinium sp.
	Gymnodinium catenatum
	Gymnodinium sanguineum
	Cochlodinium sp.
	Polykrikos kofoidii
Prorocentraceae	*Prorocentrum micans*
	Prorocentrum lima
Noctilucaceae	*Noctiluca scintillans*
Peridiniaceae	*Protoperidinium depressum*
	Protoperidinium leonis
	Protoperidinium divergens
	Protoperidinium conicum
	Protoperidinium pellucidum
	Protoperidinium sp.
	Diplopsalis sp.
Pyrocystaceae	*Pyrocystis* sp.
Pyrophacaceae	*Pyrophacus steinii*
Diatoms	
Chaetocerotaceae	*Bacteriastrum* spp.
	Chaetoceros spp.
Coscinodiscaceae	*Coscinodiscus* spp.
Rhizosoleniaceae	*Rhizosolenia* spp.
	Guinardia spp.
Melosiraceae	*Melosira* spp.
Naviculaceae	*Pleurosigma* spp.
	Navicula spp.
Bacillariaceae	*Pseudonitzschia* spp.
Thalassiosiraceae	*Skeletonema* spp.
	Thalassiosira spp.
Thalassionemataceae	*Thalassionema* spp.
	Thalassiothrix spp.
Oscillatoriaceae	*Trichodesmium* spp.
Flagellates	
Chatonellaceae (Raphidophytes)	*Chatonella*
Dictyochaceae (Chrysophytes)	*Dictyocha* spp.

TABLE 2.

Corelation analysis between *Pyrodinium* cell count and climatological conditions.

	Bataan	Cavite
Temperature	0.08389	0.3793
Rainfall	0.2418	0.0203
Wind force	0.0320	−0.0998

Peridiniaceae, *Pyrocystaceae*, and *Pyrophacaceae* were represented. The diatoms belonged to the families *Chaetocerotaceae*, *Coscinodiscaceae*, *Rhizosoleniaceae*, *Melosiraceae*, *Naviculaceae*, *Bacillariaceae*, *Thalassiosiraceae*, *Thalassionemataceae*, and *Oscillatoriaceae*. *Chatonella*, a raphidophyte, and *Dictyocha*, a chrysophyte, were the flagellates present in samples from 1999.

In the majority of the samples, diatoms such as *Skeletonema* spp., *Chaetoceros* spp., *Coscinodiscus* sp., and *Thalassiosira* sp. occurred in high densities. Dinoflagellates such as *Noctiluca scintillans*, *Protoperidinium* spp., and *Ceratium furca* dominated the dinoflagellate population. *Noctiluca scintillans* was consistently present during the three years of study (Fig. 5) with a distinct increase in population during northeast monsoon and tradewinds before the onset of rainy days. *C. furca* formed blooms during the southwest monsoon in 1999 in Bataan waters. *Goniodoma* sp. was present in Bataan waters during April and July 1999. *Noctiluca scintillans* bloomed in Bataan and the diatom *Chaetoceros* spp. in Cavite, both of which succeeded the *Pyrodinium* bloom.

Relative Frequency within Three Seasons

Northeast Monsoon

Diatoms dominated the phytoplankton community in Cavite and Bataan from 1997 to 1999. The dinoflagellates were represented in higher percentage in Bataan (17%) compared to Cavite (7%) (Fig. 3).

Tradewinds

Diatoms dominated this season in Cavite and Bataan with an increase in percentage of dinoflagellates (33%) in Bataan without an increase in Cavite (7%) (Fig. 3).

Southwest Monsoon

Diatoms dominated this season in Cavite, while in Bataan, diatoms (54%) were only slightly more dominant than dinoflagellates (46%) (Fig. 3).

***Pyrodinium bahamense* var. *compressum* Bloom**

Pyrodinium bloomed in 1998 from April to October (Fig. 4). The percentage composition of phytoplankton (Tables 3 and 4) showed that in 1998 *Pyrodinium* dominated the plankton community in Bataan, while in Cavite, the organism also bloomed but not at high enough concentrations to dominate the other phytoplankton species.

Other Potentially Harmful Algal Species

Phytoplankton composition showed the presence of other potentially harmful algal species aside from *Pyrodinium*. These harmful organisms include *Alexandrium* sp., *Gonyaulax* sp., *Gymnodinium catenatum*, *Dinophysis* spp., *Prorocentrum* spp., and *Noctiluca scintillans*. A *N. scintillans* bloom is not toxic but may cause fish kills due to oxygen depletion.

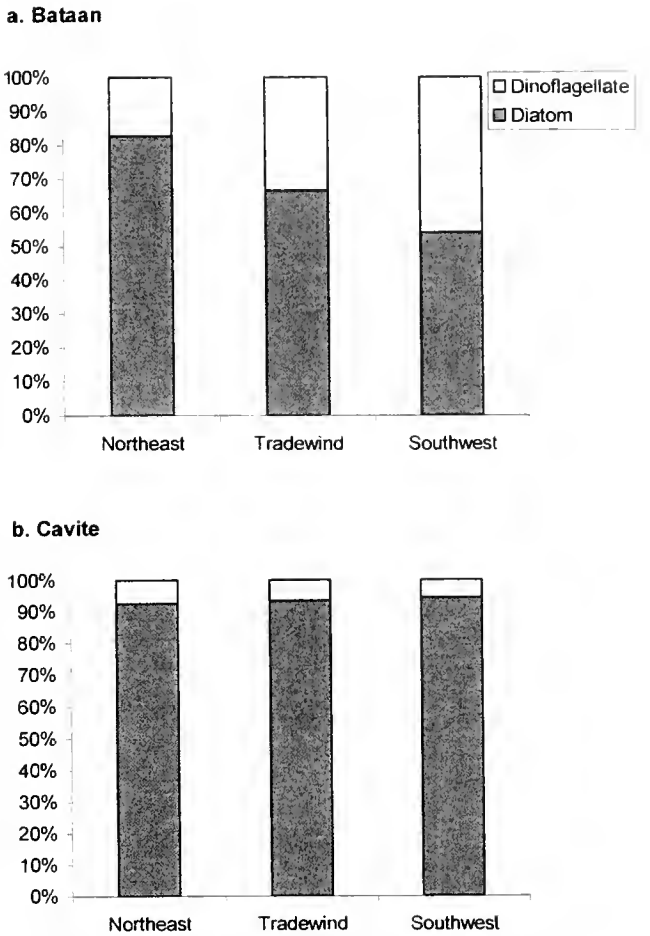

Figure 3. Percentage composition of diatoms and dinoflagellates in 3 different seasons from 1997 to 1999 (a) Bataan and (b) Cavite.

DISCUSSION

Time series data showed that diatoms dominated the phytoplankton population in Manila Bay throughout the three seasons of study (Fig. 3). Bataan and Cavite, though situated within the same bay, have distinct differences during the three seasons with respect to dinoflagellate population. There is a pronounced increase in dinoflagellate population in Bataan from northeast mon-

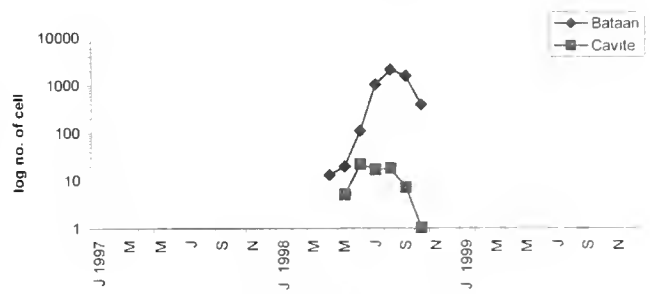

Figure 4. *Pyrodinium bahamense* var. *compressum* cell count from 1997 to 1999.

TABLE 3.

Yearly Composition of Phytoplankton in Bataan

Dinoflagellates	Percent (%) Composition per Year		
	1997	1998	1999
Pyrodinium bahamense var. *compressum*		89.58	
Alexandrium spp.			0.03
Cochlodinium spp.	12.91	0.02	0.08
Dinophysis spp.	2.00	0.27	1.54
Goniodoma spp.			1.50
Gonyaulax spp.			0.17
Gymnodinium spp.			0.10
Gyrodinium spp.			0.02
Noctiluca scintillans	36.25	5.15	13.37
Prorocentrum spp.			0.81
Protoperidinium spp.	3.14	0.44	2.41
Pyrocystis noctiluca			0.02
Pyrophacus spp.			0.26
Scripsiella spp.			0.25
Diatoms			
Bacteriastrum spp.	0.69		1.38
Ceratium spp.	4.28	0.51	33.42
Chaetoceros spp.	17.27	1.07	14.52
Coscinodiscus spp.	4.73	0.75	3.07
Dictyocha spp.		0.03	0.09
Guinardia spp.		0.14	0.59
Navicula spp.		0.03	0.02
Pleurosigma spp.	5.21	1.19	0.34
Pseudonitzchia spp.	0.41		
Rhizosolenia spp.	3.95	0.38	2.05
Skeletonema spp.			22.92
Thalassionema spp.	2.44	0.19	0.07
Thalassiosira spp.	4.48	0.22	1.00
Thallasiothrix spp.	2.24		
TOTAL	100%	100%	100%

TABLE 4.

Yearly Composition of Phytoplankton in Cavite

Dinoflagellates	Percent (%) Composition per Year		
	1997	1998	1999
Pyrodinium bahamense var. *compressum*		6.94	
Alexandrium spp.			0.01
Chatonella spp.			0.01
Cochlodinium spp.		1.36	0.01
Dinophysis spp.	1.67	1.09	0.19
Goniodoma spp.			0.40
Gonyaulax spp.			0.01
Gymnodinium spp.	0.10	3.65	0.25
Gyrodinium spp.		2.86	0.01
Noctiluca scintillans	6.75	9.30	1.02
Prorocentrum spp.		0.35	0.50
Protoperidinium spp.	1.37	2.28	1.62
Pyrocystis noctiluca			0.01
Pyrophacus spp.			0.07
Scripsiella spp.			0.19
Dictyocha spp.			0.01
Diatoms			
Bacteriastrum spp.	0.60	0.82	1.87
Ceratium spp.	2.83	1.97	2.92
Chaetoceros spp.	37.42	35.20	9.24
Coscinodiscus spp.	16.16	16.05	7.63
Guinardia spp.		0.99	0.31
Melostra spp.			0.06
Navicula spp.		0.10	
Pleurosigma spp.	5.69	3.44	0.63
Pseudonitzchia spp.	4.68	0.10	0.95
Rhizosolenia spp.	1.63	8.52	1.35
Skeletonema spp.			64.42
Thalassionema spp.	6.35	7.74	4.95
Thalassiosira spp.	13.39		0.17
Thallasiothrix spp.	1.36		1.18
TOTAL	100%	100%	100%

soon to tradewinds to southwest monsoon, while in Cavite, dinoflagellates constitute a minor portion of the phytoplankton community during the whole year.

No *Pyrodinium* cells were detected in 1997 and 1999. *Pyrodinium bahamense* var. *compressum* bloomed in 1998 during the southwest monsoon, and cells were first detected during the month of April in Bataan and in May in Cavite. *Pyrodinium* red tides in the Bay occurred at the onset of rainy season after a warm dry period similar to the observation of Bajarias and Relox (1996). Peaks of the bloom occurred in months with heavy rainfall (June to August), which was observed in 1991–1994 blooms and also in 1998 *Pyrodinium* bloom. The peak of the bloom occurred during the months of June to August and terminated in October. The 1998 *Pyrodinium* bloom, however, showed a weak relationship with the climatological factors (Fig. 5) based on correlation analysis (Table 2). In terms of rainfall, however, *Pyrodinium* bloom started at the onset of the rainy season just after a dry period (Figs. 5b, 5c), which is necessary in the initiation of *Pyrodinium* blooms as discussed by Corrales and Crisostomo (1996), Bajarias and Relox (1996), Velasquez et al. (1997), and Azanza et al. (1998).

Previous studies by Corrales and Hall (1993), Bajarias and Relox (1996), and Velasquez et al. (1997) showed the optimal environmental conditions (i.e., water temperature, salinity, rainfall, and nutrients) for the growth of *Pyrodinium* culture in the laboratory and in the field. The amount and availability of dissolved nutrients in the water column play a significant role in regulating the bloom of *Pyrodinium* (Velasquez et al. 1997).

Villanoy et al. (1996) studied the distribution patterns of *Pyrodinium* cyst density, which indicates that the onset of the algal bloom may be related to some physical, chemical, and biological factors in the bay. Manila Bay water circulation is described to have a double-gyre system where one gyre is located at the western side (Bataan) and the other is at the eastern side (Cavite) (Yniquez et al., 2000). The nonoccurrence of the *Pyrodinium* bloom in 1997 and 1999 needs to be studied further through ecophysiological experiments and field studies.

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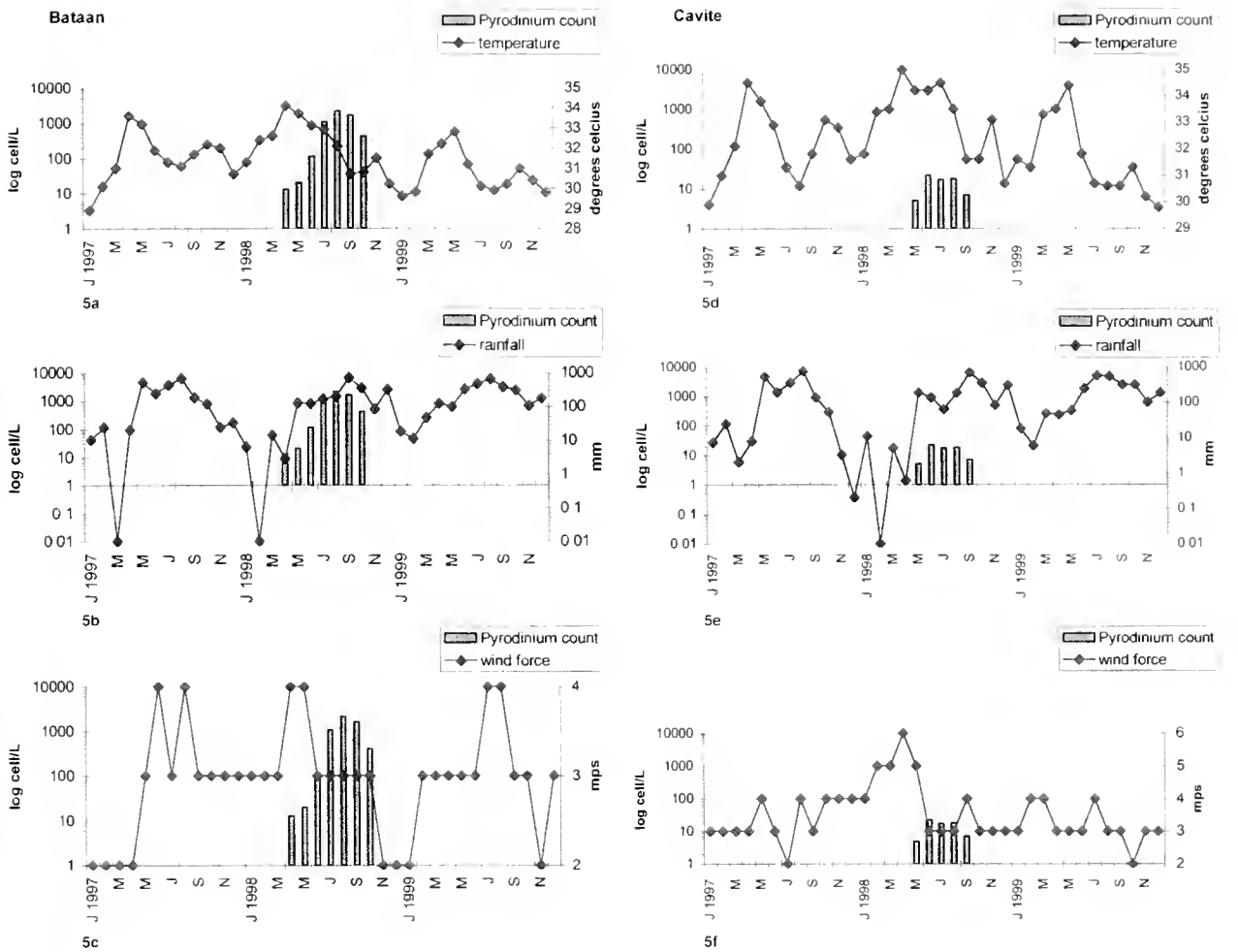

Figure 5. Climatological conditions in relation with *Pyrodinium* cell density.

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DISTRIBUTION OF *GYMNODINIUM CATENATUM* GRAHAM AND SHELLFISH TOXICITY ON THE COAST OF SUCRE STATE, VENEZUELA, FROM 1989 TO 1998

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ABSTRACT *Gymnodinium catenatum* blooms were first detected on the Venezuelan coasts in 1989. Since then, monitoring along the northeastern coast of Sucre state and the Gulf of Cariaco was carried out to detect the presence of this organism and to measure environmental parameters (temperature and salinity) that may be associated with its appearance. *G. catenatum* has been observed every year along the coast, with maximum abundance between June and October. Blooms of *G. catenatum* (> 1,000 cells · ml^{-1}) have been recorded only in the northeastern coast during the rainy season (July – Nov.) when water temperature was above 25°C and salinity was 36.5 to 38‰. The lowest abundance (< 100 cells · ml^{-1}) occurred from Dec. – June in a wide range of temperature (22–28°C) and salinity (33.2–39 ‰). The species has been less abundant in the Gulf of Cariaco and has not formed blooms yet. The dynamics of the marine environment at the north coast seem to favor the growth of *G. catenatum* populations. Shellfish toxicity showed different PSP levels and did not always coincide with the presence of *G. catenatum*.

KEY WORDS: *Gymnodinium catenatum*, shellfish toxicity, Sucre state, Venezuela

INTRODUCTION

Algal blooms in the coastal waters of northeastern Venezuela occur with yearly frequency. Many blooms are associated with toxigenic species that induce toxicity in bivalve mollusks, which can have severe impacts on public health, on the regional economy, and upon the marine environment. Ferraz-Reyes (1992) reported that red tide events off the northeastern coast of Venezuela occur mainly during the dry season and occasionally during the rainy season.

One of the species responsible for blooms along the coast of Sucre state has been *Gymnodinium catenatum* (Graham 1943). Its appearance has been associated with PSP toxicity of bivalve mollusks of commercial value (La Barbera-Sánchez et al. 1993). The species was first detected in Venezuelan waters in 1989. Since then it has reappeared several times, causing PSP on the northeastern coast of Sucre state. This athecate dinoflagellate has a widespread distribution. It has been reported from the Mexican Pacific Coast (Morey-Gaines 1982, Mee et al. 1986), the coasts of Argentina (Balech 1964), the Atlantic Coast of Spain (Estrada et al. 1984, Fraga et al. 1988), the Mediterranean Coast (Carrada et al. 1991), and Australia (Oshima et al. 1987, Hallegraeff et al. 1989).

The presence of *G. catenatum* in Venezuelan coastal waters is associated with certain environmental conditions, particularly with intrusion of warm seawater and periods of upwelling relaxation. Fraga et al. (1988, 1990) and Fraga (1996) associated the presence of *G. catenatum* with the introduction of surface warm waters from offshore toward coastal inlets during the period of upwelling relaxation after summer time. In this study, the presence and abundance of *G. catenatum* is compared with observed trends of salinity and temperature of coastal waters in Sucre state, and toxicity events during the monitoring program performed from 1989 to 1998.

MATERIALS AND METHODS

A monitoring program to detect the presence of toxic algae in the plankton and toxicity episodes in bivalve mollusks was implemented from 1989 to 1998. Water samples were taken at sea surface and 3 m deep every 15 days, in different localities along the coast of Sucre state where bivalve mollusks beds are found. Three sampling sectors were established: the northeastern sector with stations at Bahía Patilla, La Iglesia, and San Juan de Las Galdonas; the northwestern sector with two stations at Chacopata; and the southwestern sector with stations at La Chica and Peñas Blancas within the Gulf of Cariaco (Fig. 1).

Water samples were obtained using a Van Dorn bottle, with a portion fixed with Lugol solution for quantitative cell counts by the Utermöhl (1958) method. The other portion was used to measure water temperature with a thermometer (A = 1°C) and salinity with an ATAGO refractometer (A = 0.1‰). Transparency at each sampling station was measured using a Secchi disc. Phytoplankton samples were also obtained using a net with 25 μm mesh for species identification.

The concentrations of PSP toxins in ark shells (*Arca zebra*) and mussels (*Perna perna*) were measured by mouse bioassay (AOAC 1984).

RESULTS AND DISCUSSION

Gymnodinium catenatum has a wide distribution on the coast of Sucre state. The species was first found in the coastal zone of Sucre state within waters of the Gulf of Cariaco during 1986, in very low density. In April-May 1988, *G. catenatum* appeared in coexistence with *Alexandrium tamarense*, *Gonyaulax* cf. *sousa*, and *Gonyaulax polygramma*, in a bloom caused by the latter species, which caused toxicity in the mussel, *Perna perna*, cultivated in the Gulf of Cariaco (La Barbera-Sánchez et al. 1993).

In 1989, blooms of *G. catenatum* were recorded in the northeastern and northwestern sectors of Sucre state, with abundance larger than 10×10^2 cells · ml^{-1} in February and April (Fig. 2). *G. catenatum* coexisted in low abundance with *A. tamarense* and *A. monilatum*. The presence of *G. catenatum* in these sectors was associated with toxicity events of PSP, which reached levels higher than 600 μg STX/100 g meat and affected several species of bivalve mollusks of commercial value (La Barbera-Sánchez *op*

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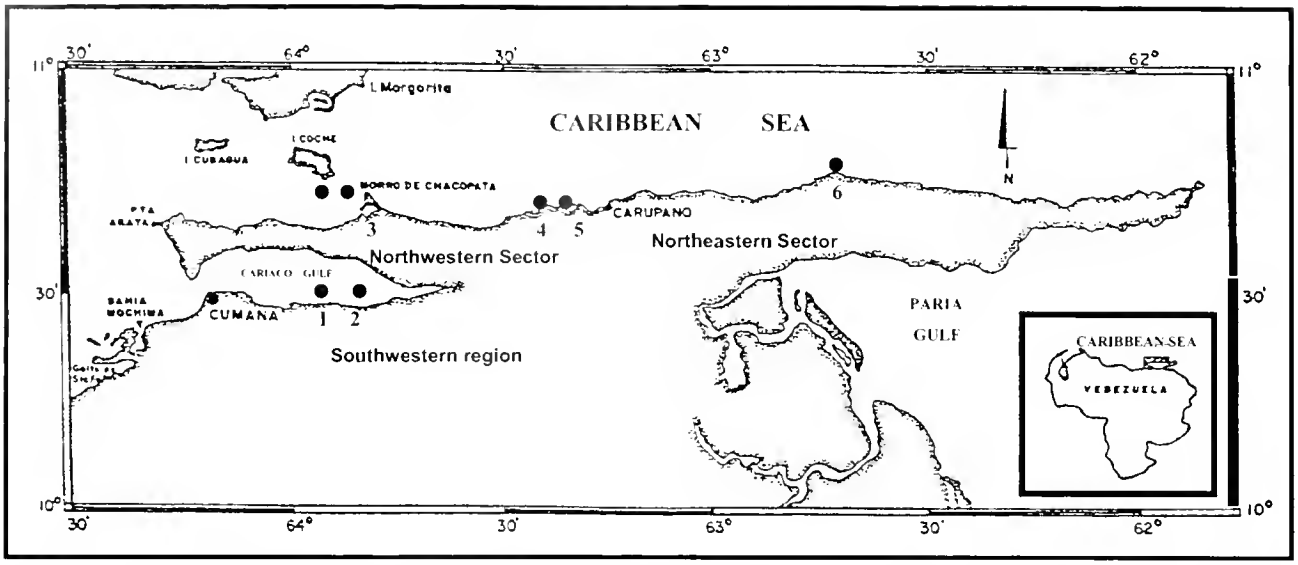

Figure 1. Location of sampling stations in Sucre state. southwestern sector: Peñas Blancas (1), La Chica (2); northwestern sector: Chacopata (3); northeastern sector: La Iglesia (4), Bahía Patilla (5), San Juan de Las Galdonas (6).

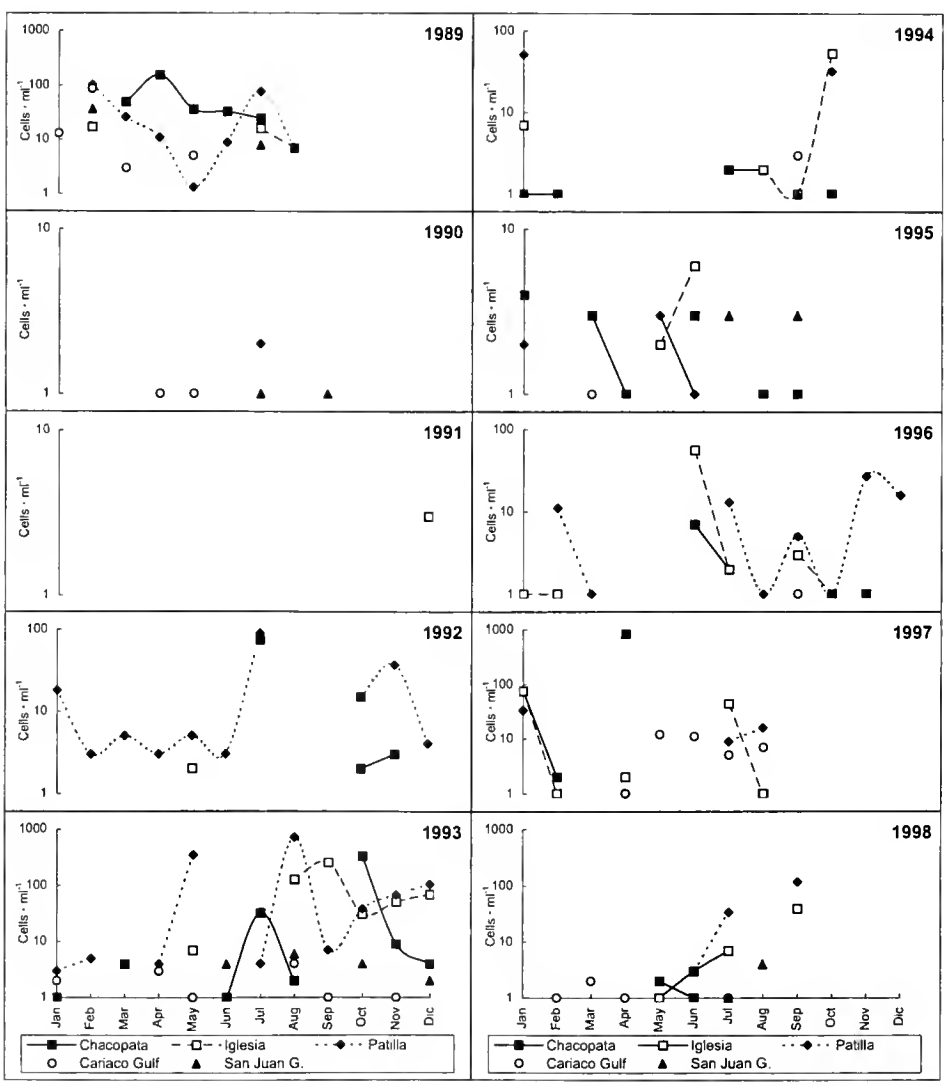

Figure 2. Abundance variation of *Gymnodinium catenatum* on the coast of Sucre state, Venezuela: 1989–1998.

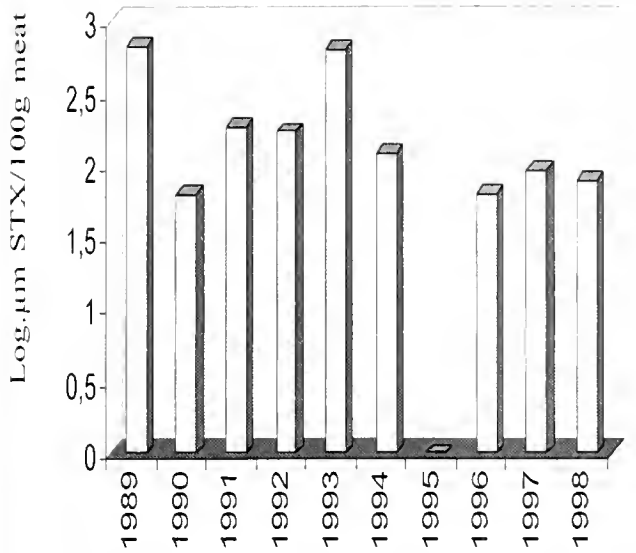

Figure 3. Variation annual mean of the toxin concentration on the coast of Sucre state.

cit.). In the northeastern sector, toxicity levels in the mussel, *P. perna*, and the clam, *Tivela mactroides*, reached values higher than 1,000 µg STX/100 g meat. The death of mussels in natural banks was also observed. In the northwestern sector, the levels of PSP toxin in ark shells, *Arca zebra*, were beyond those permitted for human consumption. These events caused intoxication by consumption of bivalve mollusks in 15 persons, and great economic impact in the region.

During the period 1990–92, *G. catenatum* was observed in low abundance (Fig. 2), but the number of observed organisms (from < 1 to 10^2) in the northeastern sector did not correspond with toxicity levels recorded in the mussel, *P. perna*, during 1991 and 1992 (Fig. 3).

In 1993, *G. catenatum* was observed during the entire year in the northeastern sector with variable densities (from 1 to 1,342 cells · ml^{-1}). The highest abundance was recorded in May, August, September, and October, while the lowest abundance was observed during the first months of the year (Fig. 2). The period of highest densities coincided with episodes of toxicity in November and December (1,255 µg STX/100g meat) that extended until October 1994, at toxicity levels of 100 µg STX/100g meat.

In 1994, *G. catenatum* registered low abundance and there was toxicity in mussels almost all year long, with maximum levels between January and April. However, there was no correspondence between the toxicity levels and the scarcity of vegetative cells of the toxic species. January and April are months of strong upwelling along the coasts off Sucre state; therefore, it is possible that the toxic event may have been originated by cysts resuspended by upwelling in the water column, as has also been pointed out by La Barbera-Sánchez and Estrella (1996).

The years 1995, 1996, 1997, and 1998 (Fig. 2) were notorious for the low number of cells of *G. catenatum*, except in the northwestern sector, where there were increases in April 1997 (984 cells · ml^{-1}) at surface level. Otherwise, the levels of toxicity for PSP stayed low, with a total absence in 1995.

For the years 1989 and 1993 the toxicity levels in bivalve mollusks of Sucre coasts were higher than the allowable maximums (> 600 µg STX/100 g meat) (Fig. 3). The toxic levels for 1990, 1996, and 1998 were lower than 80 µg STX/100 g meat. The levels for the remaining years in the study yielded values between 90 and 190 µg STX/100 g meat.

G. catenatum was always present in the area, at varying temperatures and salinities. It appears in higher density from July to February in coincidence with the rainy season (from June to November) and with the onset of trade winds and upwelling (November, December, January, and February) (Figs. 4 a, b, c).

G. catenatum recorded low densities within a wide range of temperatures (22–28°C) and salinity gradients (33.2 –39‰). Blooms of the organism (> 1,000 cells · ml^{-1}) were observed in 1989 (February), 1993 (May, August, September, and October), 1997 (January, April), and 1998 (September), in waters with temperatures above 24°C and salinity gradients between 35.5 and 38‰ during the rainy season, contrasting with the southwestern sector (Gulf of Cariaco), where the species has not bloomed yet (Fig. 4 a). The species was more abundant and frequent in the northwestern and northeastern sectors (Figs. 4 b and c). However, this behavior is more evident in the northeastern sector (Fig. 4 c), where the species has a spatial and seasonal distribution.

The northeastern sector is characterized by strong swelling and by the influence of the low salinity and influx of nutrients from the Gulf of Paria and adjacent regions (Okuda et al. 1974). It has, in addition, increased orthophosphate content during the rainy season, which favors blooms of diatoms and dinoflagellates (La Barbera-Sánchez et al. 1988). Figueiras and Pazos (1991) reported dense overgrowths of *G. catenatum* in water bodies of intense agitation, similar to what this study found for the northeastern region. Thus, the increase in the number of cells may be influenced by soil and wastes washed off by rain. This phenomenon affects the saline concentrations and incorporates organic and inorganic elements that serve as nutrient sources for the species. Peperzak et al. (1996) reported that an increase of freshwater in coastal areas may result in a transient stratification of salinity and reduced turbulence. This allows for greater stability of the water column and favors the growth of dinoflagellates. However, it has been found that it is thermal stratification, rather than salinity stratification, that leads to dinoflagellate accumulation and dominance. Likewise, La Barbera-Sánchez and Estrella (1996) found a significant correlation between proliferation of *G. catenatum* during the rainy season with temperature and transparency in the northeastern sector of the coast of Sucre.

Results have revealed that the distribution of *G. catenatum* along the coast of Sucre state does not seem to be related to the temperature and salinity. However, the abundance and the periods of toxicity do point to a transient relationship with temperature increases and rainy seasons. The dynamics of the marine environment along the northwestern and northeastern sectors in the coast of Sucre state, which need to be fully studied, seem to favor the growth of *G. catenatum* populations, perhaps due to a greater availability of nutrients or to the presence of some oligoelement in the water during the rainy season.

ACKNOWLEDGMENTS

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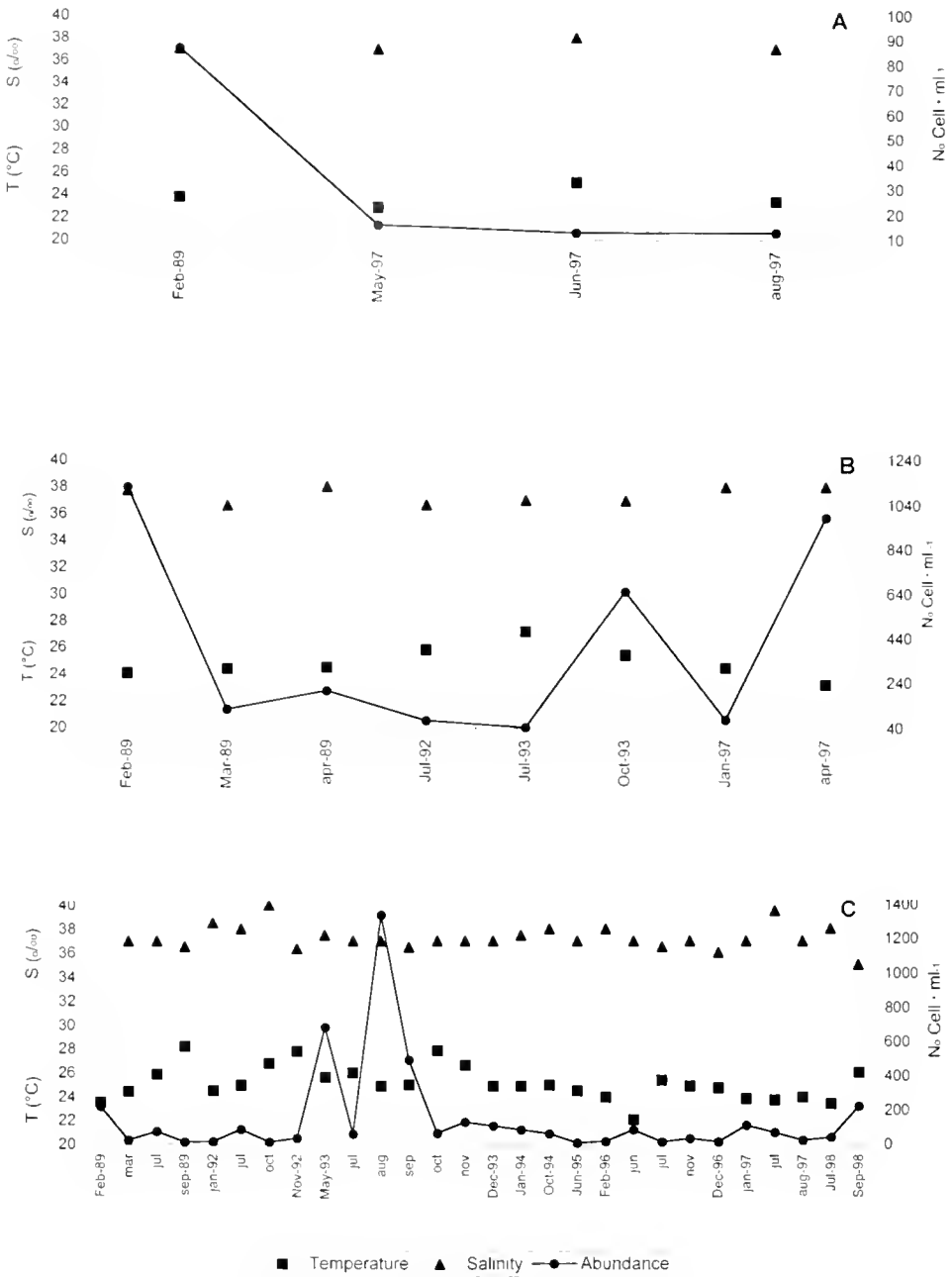

Figure 4. Relationship between *Gymnodinium catenatum* abundance with salinity and temperature in the sectors: A) southwestern, B) northwestern, and C) northeastern of Sucre state.

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PIGMENT PROFILE AND VIOLAXANTHIN CYCLE OF *HETEROSIGMA AKASHIWO* (RAPHIDOPHYCEAE)

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ABSTRACT Pigment profiles of the Raphidophycean *Heterosigma akashiwo* were analyzed by high-performance liquid chromatography (HPLC) with a diode array detector. Chlorophyll $c_1 + c_2$, fucoxanthin, violaxanthin, antheraxanthin, zeaxanthin, chlorophyll *a*, and β-carotene in three strains of *H. akashiwo* (NIES6, 10, 293) were identified. The pigment profiles of *H. akashiwo* in a light:dark cycle were also analyzed by HPLC. The peaks of antheraxanthin and zeaxanthin were higher in the light, while the reverse was recorded for violaxanthin. This is the first evidence of the existence of a violaxanthin cycle in Raphidophyceae.

KEY WORDS: Raphidophyceae, *Heterosigma akashiwo*, pigments

INTRODUCTION

Since the analysis of microalgal pigments by high-performance liquid chromatography (HPLC) was developed (Mantoura and Llewellyn 1983, Wright and Shearer 1984, Wright et al. 1991, Suzuki et al. 1993, Latasa et al. 1996, Furuya et al. 1998), the pigment profiles of many classes of microalgae have been analyzed by this method (Kohata et al. 1991, Suzuki and Ishimaru 1992, Garrido and Zapata 1998). In particular, HPLC analysis of pigments has been conducted to investigate marine microalgal species that contribute to primary production (Gieskes and Kraay 1983, 1986, Goericke and Repeta 1993, Letelier et al. 1993, Andersen et al. 1996, Bidigare and Ondrusek 1996, Roy et al. 1996, Suzuki et al. 1997). Analysis by HPLC is also useful to study the synthesis of microalgal pigments, such as in the diadinoxanthin cycle and violaxanthin cycle (Goericke and Welschmeyer 1992, Porra et al. 1997, Lohr and Wilhelm 1998, Oku and Kamatani 1999). In recent years, several reports (Fiksdahl et al. 1984a, Fiksdahl et al. 1984b, Yamaoka and Takimura 1985, Kohata and Watanabe 1988, Kohata et al. 1991) and a review (Jeffrey and Vesk 1997) of pigment profiles of harmful algae of the family Raphidophyceae have been published. It is interesting that these profiles, in particular that of *Heterosigma akashiwo*, differ among the reports (Fiksdahl et al. 1984b, Yamaoka and Takimura, 1985, Kohata et al. 1991), suggesting variation in the pigment profiles of such species.

In the present study, detailed pigment profiles of three strains of *H. akashiwo* were obtained and the violaxanthin cycle of this species was investigated.

MATERIALS AND METHODS

Pigment Analysis by HPLC

The following marine microalgae with known major pigments were cultured: Rhodophyceae, *Porphyridium cruentum* (CSIRO25); Bacillariophyceae, *Phaeodactylum tricornutum* (CSIRO29); Chlorophyceae, *Dunaliella tertiolecta* (CSIRO175); and Dinophyceae, *Amphidinium cartererae* (CSIRO212), all obtained from the CSIRO Microalgae Research Centre, CSIRO Marine Research Laboratories, Tasmania. Culture conditions were: temperature 21 ± 1°C, illumination 8000 ± 1000 lux and a light: dark cycle of 14:10 hours. Test tubes (25 × 200 mm, 64 ml) containing 30 ml of f/2 medium (Guillard and Ryther 1962) were used as the culture vessels.

Thirty milliliters of the growth phase medium were filtered on glass-fiber filters (Whatman GF/F). The filters were then extracted with 10 ml *N, N* -dimethylformamide (DMF) (Suzuki and Ishimaru, 1990). Chlorophyll *a* standard (Chl *a*; Wako Pure Chemicals, Ltd.) was also dissolved in DMF. Each algal sample extract and the Chl *a* standard were chromatographed on an STR ODSII column (Shimadzu Chemical Industries, Ltd.) with a Shimadzu Raphidophyceae HPLC system (system controller, SCL-10AVP; auto injector, SIL-10ADVP; liquid chromatograph, LC-10ATVP; column oven, CTO-10AVP; and diode array detector, SPD-M10AVP). The temperature of the column was kept at 40°C. The HPLC solvent system followed the modified method of Suzuki et al. (1997) as shown in Table 1.

After pigment separation by HPLC, individual pigments were identified from published data of their diode array spectra (Jeffrey et al. 1997).

Analysis of Pigment Distribution in Three Strains of H. akashiwo

Three strains of *H. akashiwo* (NIES6, NIES10, NIES293) obtained from the Microbial Culture Collection, National Institute for

TABLE 1.

HPLC solvent system program

Time (min)	Flow rate (ml/min)	%A1	%B1	Conditions
0	1.0	100	0	Injection
1	1.0	0	75	Linear gradient
8.5	1.0	0	100	Linear gradient
28.5	1.0	0	100	Equilibration
28.51	1.0	100	0	
38.50	1.0	100	0	Equilibration

1. = 80:20 methanol: 0.5M ammonium acetate (pH 7.2, v/v)
2. = 70:30 methanol: acetone

*Corresponding author.

Environmental Studies (NIES Collection), Environment Agency, Japan, were cultured and pigments analyzed using procedures as mentioned above. Identification of the pigments was made by comparison of their retention time with standard and diode array spectra of pigments of the other cultured microalgae.

Response of Pigments of H. akashiwo to a Light:Dark Cycle (Light Intensity)

H. akashiwo NIES6 was pre-cultured in three test tubes with 30 ml of f/2 medium. 30 ml of medium from the exponential growth phase was added to three 300 ml glass Erlenmeyer flasks containing 200 ml of f/2 medium. The microalgae were cultured under illumination of 8000±1000 lux with a light:dark cycle of 14:10 hours for four days.

The microalgae were harvested in their growth phase after 11 and 13 hours of light period (L11 h, L13 h), 10 hours of dark period (D10 h), 4 and 11 hours in the next light period (NL 4 h and NL 11 h) and 8 hours in the next dark period (ND 8 h).

The samples were frozen at −80°C immediately after filtering the cultures using glass-fiber filters. After the samples were harvested, they were extracted in DMF for 24 hours and analyzed by HPLC.

RESULTS AND DISCUSSION

Pigment Analysis by HPLC

The absorbance maxima (nm) of the pigments (Table 2) were close to those reported by Jeffrey et al. (1997). This also indicates that there was no significant influence of variations in the extraction solvent and HPLC mobile phase on the spectra of the pigments.

Analysis of Pigment Profiles in the Three Strains of H. akashiwo

The major pigments of the three strains of *H. akashiwo* (NIES6, 10, 293) were eluted from the column in the order: chl c_1 + c_2, fucoxanthin, violaxanthin, antheraxanthin, zeaxanthin, chl *a*, and β-carotene (Fig.1). The major pigments of Raphidophyceae, in particularly *H. akashiwo*, have varied among the published reports. Jeffrey and Vesk (1997) reported that the major pigments in Raphidophyceae are fucoxanthin, violaxanthin, and β,β-carotene, while Fiksdahl et al. (1984a) mentioned that the major pigments of

TABLE 2.

Spectrum and retention time of several carotenoids extracted in DMF.

Pigment	Retention time (min)	Absorbance maxima (nm)					
		Observed			Literature (Jeffrey *et al.* 1997)*		
Peridinin (*Amphidinium carterae*)							
	10.00	472			474		
	09.98	465					
	10.00	471					
Fucoxanthin (*Phaeodactylum tricornutum*)							
	10.67	450	—		449	468	
	10.67	448	—				
	10.68	449	—				
Neoxanthin (*Dunaliella tertiolecta*)							
	10.87	413	437	465	413	439	468
	10.88	413	437	465			
	10.89	413	437	465			
Violaxanthin (*Dunaliella tertiolecta*)							
	11.21	415	439	470	417	441	471
	11.21	417	440	469			
	11.22	416	440	470			
Diadinoxanthin (*Phaeodactylum tricornutum*)							
	11.68	—	446	476	427	448	477
	11.68	—	446	476			
	11.69	—	446	476			
Antheraxanthin (*Dunaliella tertiolecta*)							
	11.98	—	446	473	421	444	471
	11.99	—	446	474			
	12.00	—	446	474			
Diatoxanthin (*Phaeodactylum tricornutum*)							
	12.51	—	451	479	427	454	482
	12.52	—	453	480			
	12.53	—	452	481			
Zeaxanthin (*Porphyridium cruentum*)							
	12.83	—	451	479	428	455	483
	12.82	—	452	479			
	12.81	—	452	479			

Extraction in 90% acetone

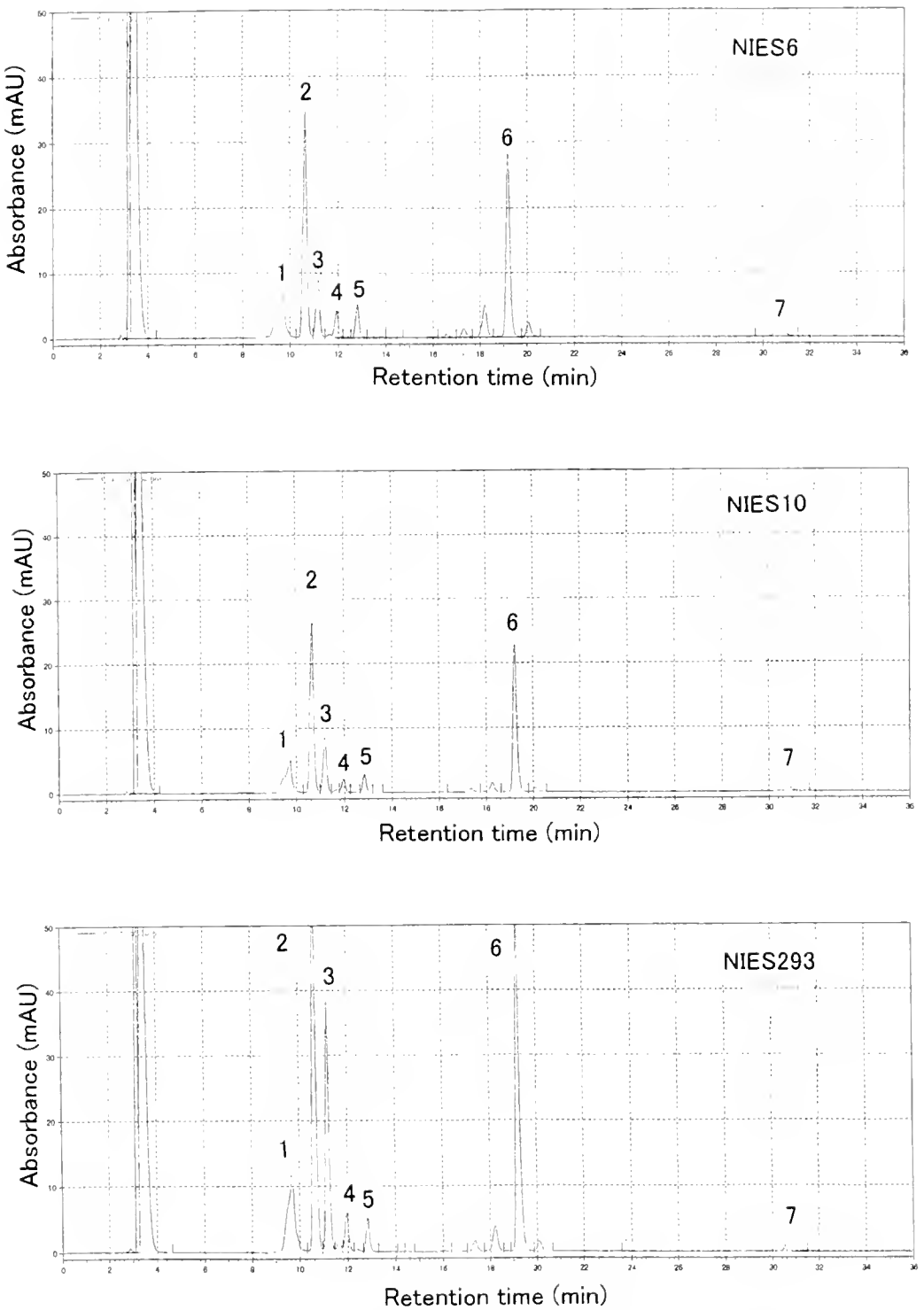


Figure 1. Typical chromatograms (435 nm) of three strains of *H. akashiwo* (NIES6, 10, 293). Individual peaks are (1) Chl c_1+c_2, (2) fucoxanthin, (3) violaxanthin, (4) antheraxanthin, (5) zeaxanthin, (6) Chl *a*, and (7) β-carotene.

Raphidophyceae are chl *a*, c_1 + c_2, fucoxanthin, violaxanthin, zeaxanthin, and β-carotene. Fiksdahl et al. (1984b) also reported that the major carotenoid in *H. akashiwo* is peridinin; fucoxanthin was not observed in their study. Yamaoka and Takimura (1985) observed that in *H. akashiwo*, fucoxanthin and β-carotene are present as major pigments while zeaxanthin was absent. Carotenoids of *C. antiqua* were fucoxanthin, violaxanthin, and β-carotene in Kohata and Watanabe's work (1988). Kohata et al. (1991) reported that fucoxanthin, violaxanthin, antheraxanthin, zeaxanthin, and β-carotene were the major carotenoids of *H. akashiwo*. The pigment

profile of *H. akashiwo* in the present experiment was similar to that reported by Kohata et al. (1991).

Response of Pigments of H. akashiwo to a Light:Dark Cycle (Light Intensity)

The peaks of antheraxanthin and zeaxanthin (L11 h, L13 h, NL4 h and NL11 h) in the light were higher than those obtained under conditions of darkness (D10 h and ND8 h) (Fig.2). The reverse, however, was the case for violaxanthin. It is known that pigment profiles in Bacillariophyceae and Dinophyceae vary with light intensity. There is the so-called "diadinoxanthin cycle" in which diatoxanthin is epoxidized to diadinoxanthin in darkness and de-epoxidized from diadinoxanthin to diatoxanthin in the light (Goericke and Welschmeyer 1992, Lohr and Wilhelm 1998). The "violaxanthin cycle" is another reported cycle relating to photo-

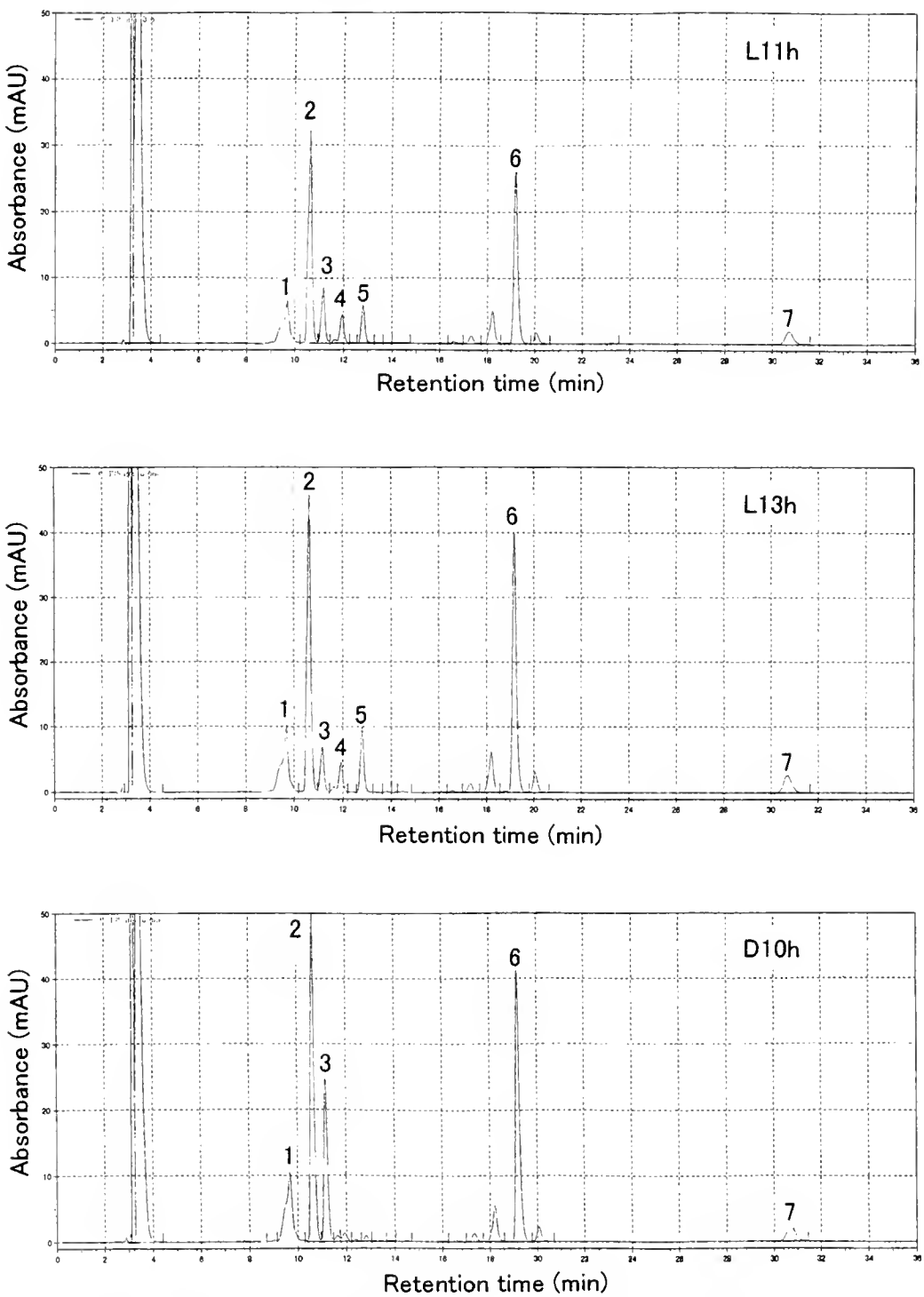
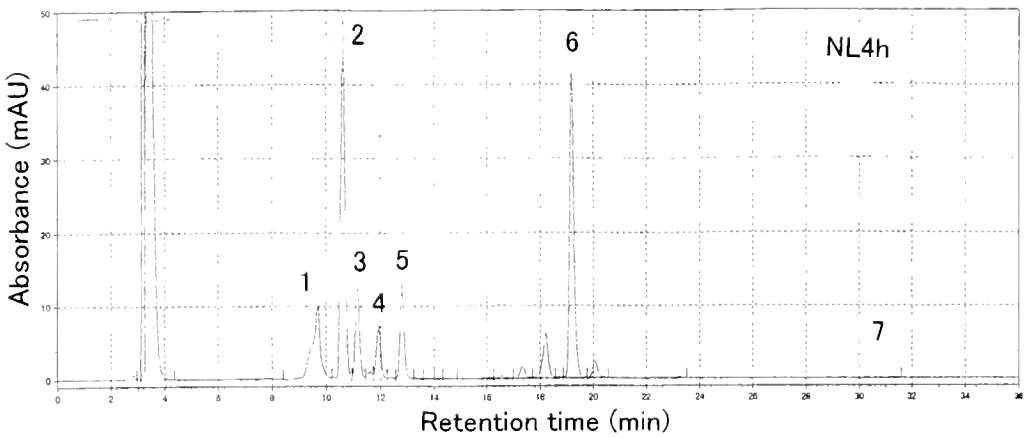
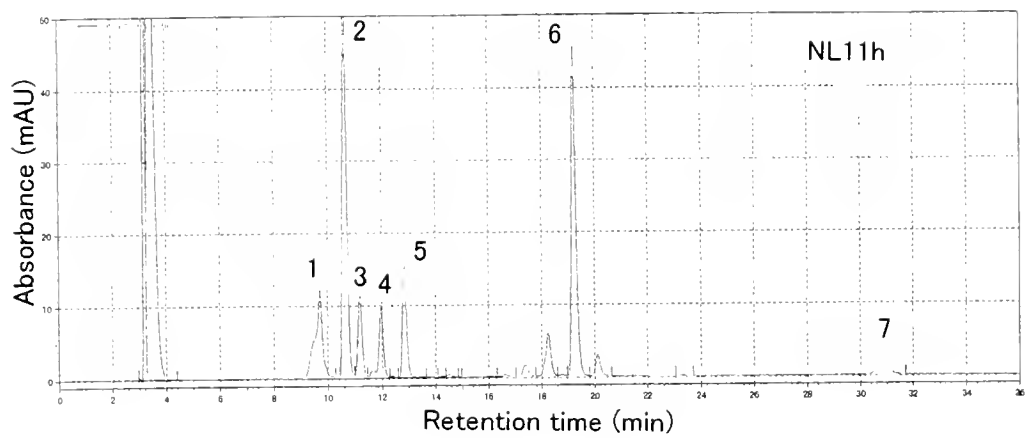


Figure 2. Typical chromatograms (435 nm) of *H. akashiwo* (NIES6) under a light:dark cycle. Individual peaks are (1) Chl $c_1 + c_2$, (2) fucoxanthin, (3) violaxanthin, (4) antheraxanthin, (5) zeaxanthin, (6) Chl *a*, and (7) β-carotene.





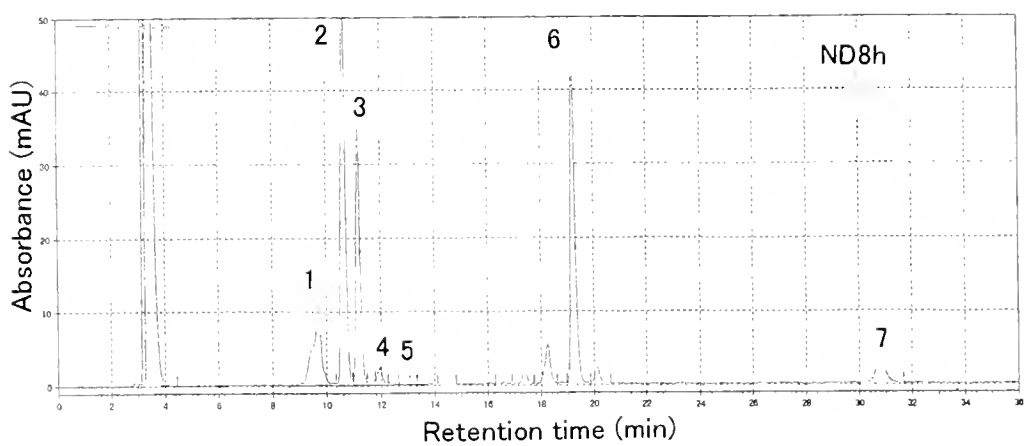


Figure 2. Continued.

chemical epoxidation : zeaxanthin and antheraxanthin are epoxidized to antheraxanthin and violaxanthin, respectively, in the dark, and these epoxidized materials are de-epoxidized to the original materials in the light (Porra et al. 1997, Lohr and Wilhelm 1999). Although diadinoxanthin and violaxanthin cycles have been observed in Bacillariophyceae, Xanthophyceae, Haptophyceae, and Dinophyceae (e.g., Willemoes and Monas 1991, Arsalane et al. 1994, Oku and Kamatani 1999, Lohr and Wilhelm 1999), the violaxanthin cycle in *H.akashiwo* has not previously been reported. Our observation is the first evidence of the existence of the violaxanthin cycle in Raphidophyceae.

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IMPACTS OF THE HARMFUL DINOFLAGELLATE, *HETEROCAPSA CIRCULARISQUAMA*, ON SHELLFISH AQUACULTURE IN JAPAN

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ABSTRACT In the last decade, the bloom-forming dinoflagellate *Heterocapsa circularisquama* Horiguchi has caused red tides along the Japanese coast. Because this dinoflagellate shows a detrimental effect on molluscan shellfishes such as bivalves and gastropods, almost all red tides have associated catastrophic death of farmed animals. Recent proliferations of *H. circularisquama* throughout the western Japanese coast have devastated the shellfish aquaculture industries and are a cause for concern due to the consequent economic losses.

KEY WORDS: *Heterocapsa circularisquama*, red tide, shellfish, death, toxicity

INTRODUCTION

Current proliferation of harmful algal blooms causes serious problems for public health and fisheries industries (Okaichi 1989, Smayda 1990, Shumway 1990, Hallegraeff 1993, Anderson 1994, Honjo 1994). On the Japanese coast, the novel marine dinoflagellate *Heterocapsa circularisquama* Horiguchi (Horiguchi 1995), appeared in 1988 and then rapidly expanded over the western area (Matsuyama et al. 1996). Red tides due to *H. circularisquama* have damaged shellfish aquaculture in most of the region (Yamamoto & Tanaka 1990, Yoshida & Miyamoto 1995, Matsuyama et al. 1996, Etou et al. 1998). Although *H. circularisquama* blooms mainly affect shellfisheries aquaculture, no harmful effects on wild and cultured finfish, other marine vertebrates, and public health hazards were recorded. Incidence of blooms of this species has increased recently, and the economic losses in aquaculture have been a cause for concern for the shellfisheries industry and society (Matsuyama et al. 1996). In the present article, we review the damage caused to aquaculture and the toxicity of the organism.

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DAMAGE TO SHELLFISHERIES AND TOXICITY

Red Tide and Shellfish Damage in Aquaculture Industries

The first incidence of red tide due to *H. circularisquama* and subsequent death of shellfish occurred in Uranouchi Bay, on the southern part of Shikoku Island (Fig. 1) in 1988. Red tide due to

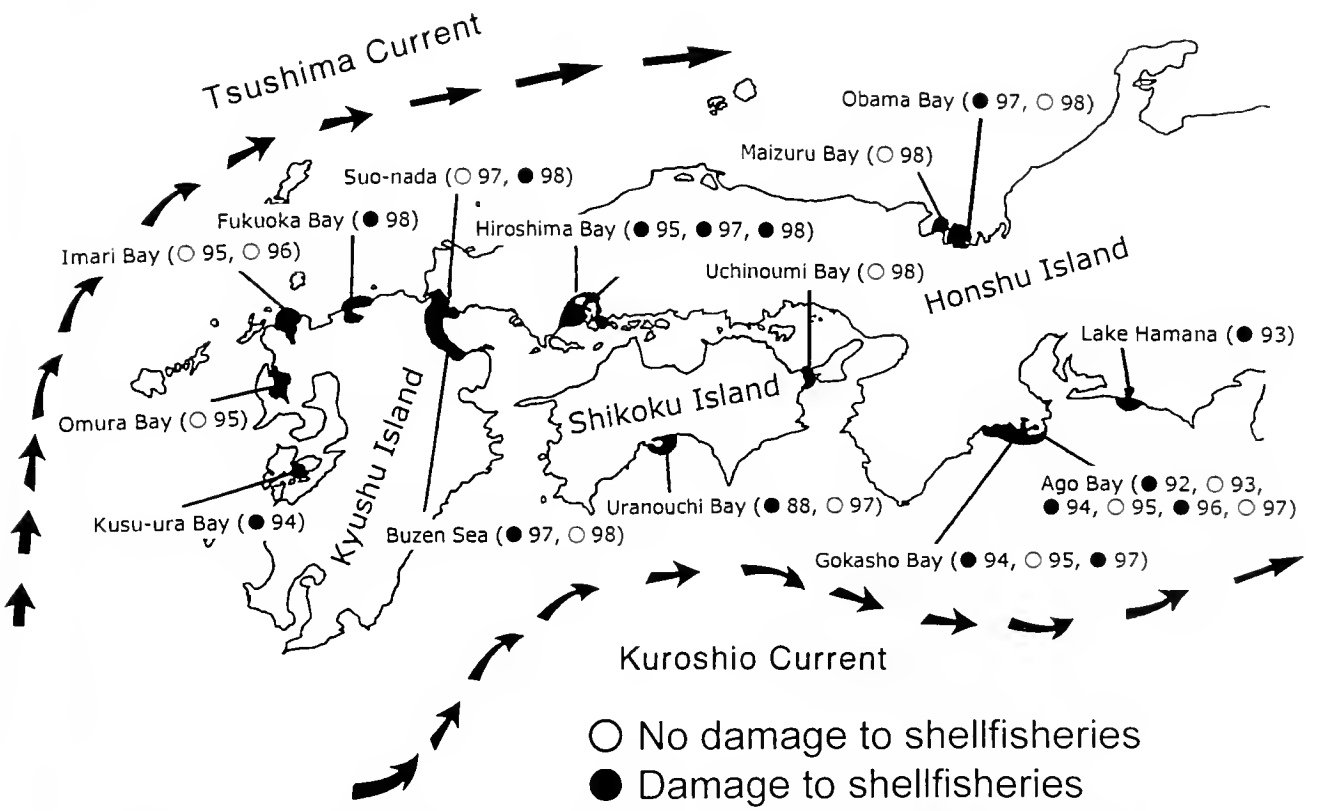

Figure 1. Records of red tide (>10^6 cells L^{-1}) due to *Heterocapsa circularisquama* and two major currents in Japan.

H. circularisquama has also been recorded at Fukuoka Bay in 1989, and at Ago Bay in 1992, resulting in mass mortality of shellfish (Fig. 2).

Until 1998, 28 cases of *H. circularisquama* red tide (maximum cell density $\geq 10^6$ cells L^{-1}), including 14 incidences leading to fisheries damage, had been recorded in 14 locations of western Japan (Fig. 1). The red tide due to *H. circularisquama* has been associated with massive killing of commercially important bivalve species: manila clam *Ruditapes philippinarum*, Pacific oyster *Crassostrea gigas*, pearl oyster *Pinctada fucata*, blue mussel *Mytilus galloprovincialis*, etc. The current proliferation of *H. circularisquama* throughout western Japan has essentially destroyed molluscan shellfish aquaculture. Economic losses of shellfish aquaculture have been estimated to be at least 93 million US dollars in the last decade (Table 1).

On the other hand, there have been no records of death of finfish and crustacean species or public health hazard due to the consumption of shellfish and other seafood products in association with the red tide of *H. circularisquama*. This type of biohazard in marine animals is markedly different from previous reports of damage caused by harmful algae responsible for PSP, DSP, ASP, NSP, ciguatera poisoning, and ichthyotoxicity.

Behavior of Shellfish Affected by H. circularisquama

The effects of *H. circularisquama* on bivalve molluses have been described in previous studies. Matsuyama et al. (1996) have observed that exposure of pearl oysters to $5–10 \times 10^6$ *H. circularisquama* cells L^{-1} resulted in death within several days, although the level of dissolved oxygen was not critical. The dead individuals have been characterized by a marked shrinkage of the mantle, decrease of glycogen lobe attached to the mantle, and gut discoloration. The symptoms clearly reflect the direct cytotoxic effect of *H. circularisquama* on pearl oyster physiology. Similar harmful effects on the oyster *C. gigas* and the mussel *M. galloprovincialis* have been shown during the red tides which have occurred elsewhere (Etou et al. 1998, Matsuyama et al. 1998).

Laboratory Exposure Experiment Using Cultured Material

Nagai et al. (1996) have shown that the mortality of pearl oyster spat caused by *H. circularisquama* depends on the cell density of this alga. Pearl oysters exposed to *H. circularisquama* cells at a density above 10^7 cells L^{-1} have showed vigorous contraction of the mantle and gills, clapping, sustained valve closure, paralysis, and heartbeat stoppage within 24 hours. Furthermore, the mussel

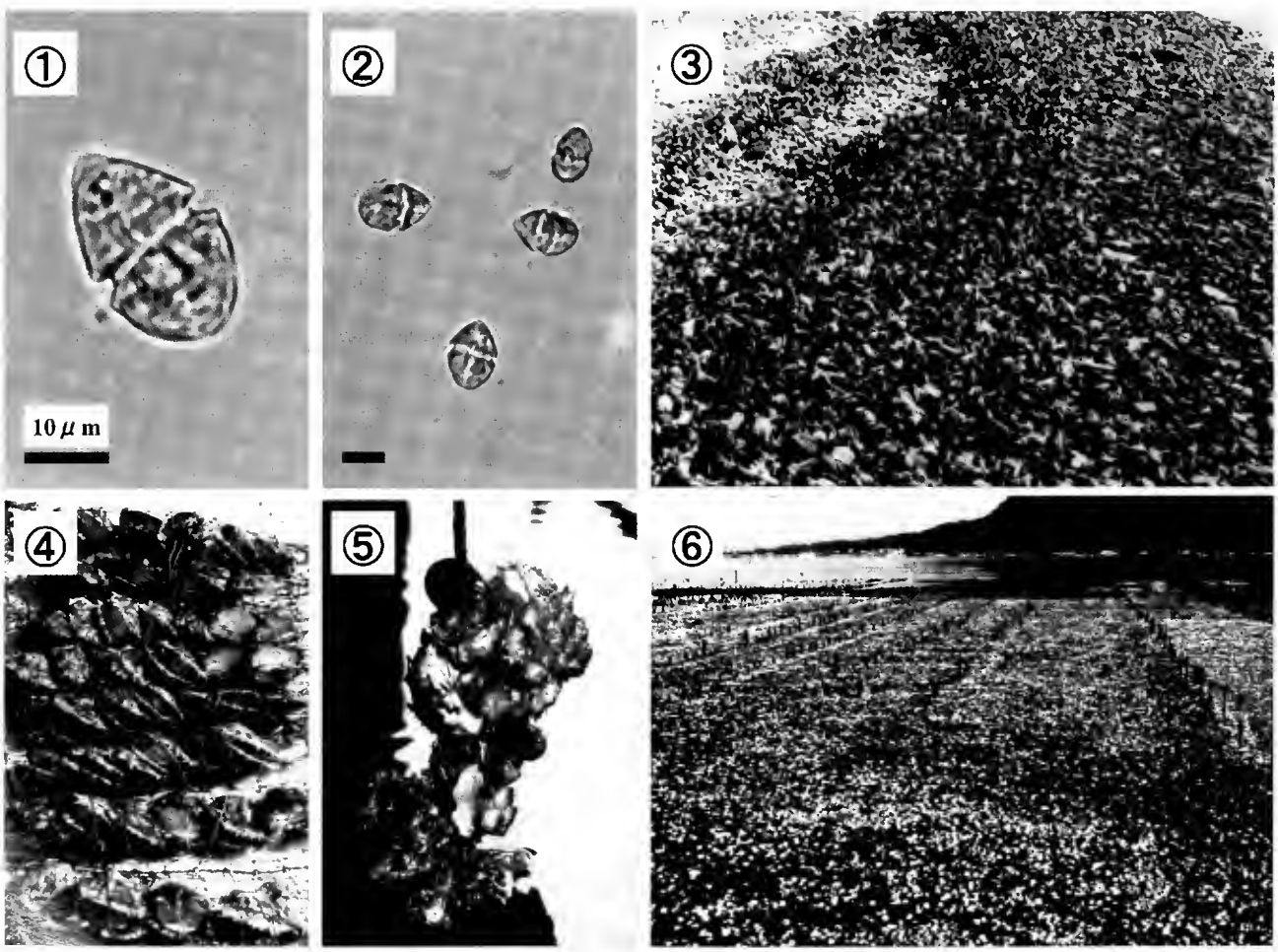

Figure 2. Photographs of *Heterocapsa circularisquama* and affected shellfish. (1) and (2): Light micrographs of *H. circularisquama;* (3) Dead shellfish: mussel *Mytilus galloprovincialis*, razor clam *Solen strictus*, manila clam *Ruditapes philippinarum* due to red tide of *H. circularisquama* (Fukuoka Bay, 1989, photograph is provided by Y. Tanaka); (4) Dead pearl oyster *Pinctada fucata* (Ago Bay, 1992); (5) Dead oyster *Crassostrea gigas* (Hiroshima Bay, 1995); (6) Farming-ground of manila clam *Ruditapes philippinarum* affected by the red tide of *H. circularisquama* (Hiroshima Bay, 1995, Photograph is provided by T. Hamasaki).

TABLE 1.

Damage to fisheries industries caused by harmful algal blooms in Japan.

Causative Agents	Periods	Amount (million $US)
Chattonella spp.	1969–1999	185
Heterocapsa circularisquama	1988–1999	92
Gymnodinium mikimotoi	1972–1999	83
Heterosigma akashiwo	1972–1999	15

M. galloprovincialis significantly reduced its feeding activity when exposed to 10^4 cells L^{-1} of *H. circularisquama*, but not in culture with 10^6 cells L^{-1} of the morphologically similar dinoflagellates *Scrippsiella trochoidea* and *Heterocapsa triquetra* (Matsuyama et al. 1997). Some harmful algae are known to be toxic to marine shellfish. The blooms associated with the unarmored dinoflagellate *Gyrodinium aureolum* and picoplankton *Aureococcus anophagefferens* referred to as "brown tide" lead to considerable failure in mussel and scallop farming (Tangen 1977, Shumway 1990). Laboratory-rearing experiments using these algae have proven considerable detrimental effects on various bivalve species. The harmful effect of *H. circularisquama* on bivalves is very specific and pronounced compared to other harmful algal species.

Harmful Effects of H. circularisquama *on Other Organisms*

Laboratory exposure experiments show that various marine animals such as bivalves, gastropods, solitary acidians, and jellyfish are affected by *H. circularisquama*, unlike vertebrates, crustaceans (lobster, shrimp, crab), starfish, and sea urchins (Table 2). On the other hand, although the occurrence of illness associated with the consumption of bivalves that accumulated *H. circularisquama* cells may be a cause for concern in humans, shellfish poisoning has never been observed in samples collected from red tide areas. Direct HPCL analysis has failed to detect PSP toxins or DSP toxins in the cells of *H. circularisquama*. No death or symptoms have been observed in 5 mice to which a cultured cell pellet of *H. circularisquama* had been injected in intraperitoneally at a rate of 10^6 cells/mouse (Table 2).

TABLE 2.

Effects of *Heterocapsa circularisquama* on various animals.

Animals	cells L^{-1}	Symptoms
Bivalves	10^4–10^5	Feeding inhibition
	$>10^6$	death
Gastropods	10^4–10^5	Unusual locomotion
	$>10^6$	death
Acidians	$>10^6$	Feeding inhibition
Jellyfish	$>10^6$	Tentacle shrinkage
Protozoa[1]	10^5–10^6	Feeding inhibition
	$>10^6$	Death
Dinoflagellates[1]	$>10^6$	Death by cell contact
Diatoms	$>10^7$	–
Copepods	$>10^7$	–
Finfish	$>10^6$	–
Crab	$>10^7$	–
Lobster, shrimp	$>10^7$	–
Lobster, shrimp	$>10^7$	–
Gammarid	$>10^7$	–
Star fish	$>10^7$	–
Sea urchin	$>10^7$	–
Mouse[2]	–	–

–: not affected

[1] species-specific

[2] intraperitoneal injection (10^6 cells/mouse)

Characterization of Heterocapsa circularisquama *Toxicity*

The toxicity of *H. circularisquama* to bivalves has been shown to be mediated by a chemical agent. The toxic effect of *H. circularisquama* on bivalves is not due to extracellular metabolites, cell exudates, and "naked cells" prepared by sonication and centrifugation (Fig. 3A). Furthermore, trypsin and SDS (sodium dodecyl sulfate) treatments have been found to decrease drastically the toxicity of *H. circularisquama* cells (Fig. 3B). Therefore, a labile protein-like complex localized on the cell surface presumably exerts a detrimental effect on bivalves. However, purification and characterization of toxic fractions have not been successful because this agent is highly labile under neutral conditions. Recently, exposure of larval manila clam to *H. circularisquama* has demonstrated acute toxicity on their survival and development, which is associated with a significant increase of intracellular calcium concentration.

SUMMARY

Blooms of the dinoflagellate *H. circularisquama* have been associated with mass mortalities of various bivalves in western Japan since 1988. Toxicity of this species is acute and specific to

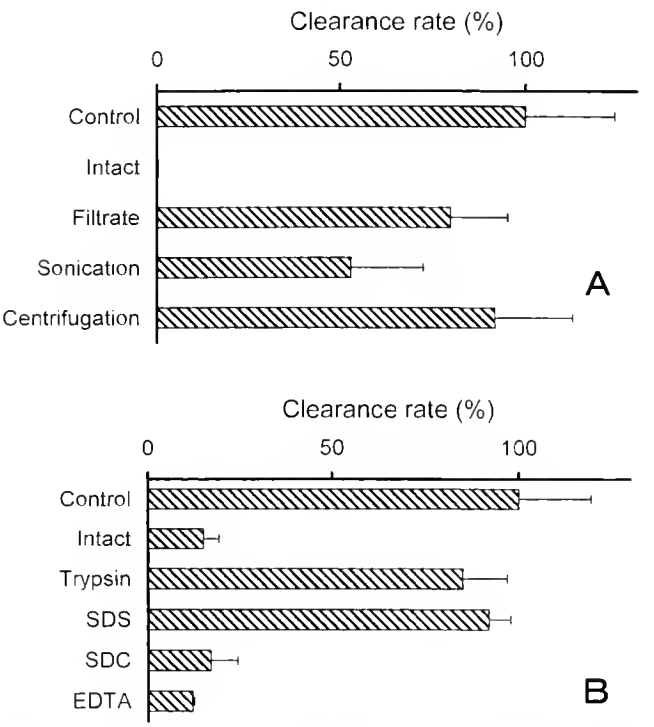

Figure 3. Relative clearance rates of *Mytilus galloprovincialis* feeding to physically (A) and chemically (B) treated *Heterocapsa circularisquama* cells (Control: *Isochrysis galbana*, 8×10^7 cells L^{-1}). Initial cell density of *H. circularisquama* ranged from $2.5–2.8 \times 10^5$ cells L^{-1}. SDS: sodium dodecylsulfate. SDC: sodium deoxycholate. DTT: dithiothreitol. Each chemical was used at a concentration lower than that which would inhibit the swimming of *H. circularisquama*. Error bars show ±S. D.

bivalves and gastropods. Unfortunately, no successful damage prevention strategy has been developed until now. Therefore, relocation and/or early harvest of organism are the only methods to reduce the shellfisheries damage. In some locations, early warning systems by local governments that are based on frequent field monitoring of *H. circularisquama* cells have successfully reduced the shellfisheries damage. *H. circularisquama* blooms do not cause finfish killing and mammals illness. However, a rapid decline of demand for products due to misinformation, rather than actual shellfish poisoning, could occur. On the other hand, toxicity of *H. circularisquama* can easily be reduced when the cells are disturbed with physical and chemical treatments (unpublished). This fact will provide crucial information for improvement of damage prevention in the future.

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GILL STRUCTURE, ANATOMY AND HABITAT OF *ANODONTIA EDENTULA*: EVIDENCE OF ENDOSYMBIOSIS

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ABSTRACT Surveys and interviews were conducted to determine sources and habitat of *Anodontia edentula*. Results showed that they inhabit muddy substrate of mangrove areas or the adjacent mudflats, burying at 20–60 cm deep in the mud. They are strategically situated in the sulfide-rich, low-oxygen layer of the substrate but have access to oxygen through their inhalant tube; both sulfide and oxygen are essential for their survival. Study of the clam's gross anatomy revealed thick, fleshy, deep purple to blackish brown gills; reduced digestive structure; and a highly elastic foot capable of extending several times longer than its body length. These observations conform with the anatomy of fellow lucinid clams. Furthermore, scanning electron micrographs showed coccoid or spherical bacteria occupying bacteriocytes in the clam's gills. Intermediate cells separating bacteriocytes observed in other lucinids were also noted in the SEM.

KEY WORDS: *Anodontia edentula*, anatomy, bacteria, gill, endosymbiosis, habitat

INTRODUCTION

Anodontia edentula (Linné, 1758) is one of the popular bivalves in the coastal areas of central and southern parts of the Philippines. It grows to a maximum size of 8–9 cm shell length (SL), 180–210 g total weight (TW) and is an important source of food and livelihood in coastal areas where it is abundant (Lebata 2000). It is one of the few expensive bivalves in the region as it is being sold per piece and not per kg or container like the common bivalves, i.e. mussels, oysters and scallops. It is a local favorite because of its sweet taste, especially when eaten raw, and men deeply believe in its aphrodisiac properties. Its popularity led to overexploitation as manifested by the decline in catch, in terms of quantity and size, through the years.

A. edentula inhabits sandy-muddy substrate near mangrove areas or the adjacent mudflats (Sotto & von Cosel 1982). It belongs to order Veneroida, family Lucinidae (Poutiers 1998), to which eulamellibranchs containing symbiotic bacteria predominantly belong (Schweimanns & Felbeck 1985).

Animal-bacteria symbioses have been observed in at least five animal phyla but are most widespread among the bivalves (Distel 1998). Symbioses have been observed in marine mollusks thriving in organically rich sediments (Janssen 1992) and hydrogen sulfide-rich habitats such as anoxic basins (Felbeck et al. 1981); seagrass beds (Cavanaugh 1983); mangrove swamps (Vetter 1985, Frenkiel et al. 1996); and sewage outfalls (Felbeck et al. 1981). Numerous studies have been conducted on this aspect in other lucinid species (Berg & Alatalo 1984, Giere 1985, Schweimanns & Felbeck 1985, Vetter 1985, Reid & Brand 1986, Distel & Felbeck 1987, Frenkiel et al. 1996, Gros et al. 1997, Gros et al. 1998). Studies on *A. edentula* have been limited to species identification and description (Sotto & von Cosel 1982, Poutiers 1998), light microscopic observation (Janssen 1992), paleontology (Cooper 1996), and just recently its elemental sulfur content (Lebata 2000).

In the Philippines, the first article on clam symbiosis was presented by Janssen (1992). Common bivalve species such as *Codakia tigerina*, *A. edentula*, and *Fimbria fimbriata* were obtained from the Mactan Island fish market and observed under a light microscope. Samples were fixed and brought to Germany for light and electron microscopy. However, only *F. fimbriata* was studied in detail using electron microscopy. Symbiosis was discussed based on literature review but no actual field observation or laboratory experiments were conducted to prove this in *A. edentula*. A new study conducted by Lebata (2000) showed that the clams have elemental sulfur in their gills. Elemental sulfur, according to Vetter (1985) is stored in the periplasmic spaces of the bacteria and represents a novel inorganic energy reserve for animal-bacteria symbiosis in the absence of sulfide. This may therefore support the claim of Janssen (1992) on the existence of endosymbiosis in this lucinid species.

Considering its potential for aquaculture and the declining state of its population, it is necessary to have a clear understanding of this clam-bacteria relationship before undertaking culture studies in this species. The objectives of this study were to describe the habitat of the clam; to study its gross anatomy and relate it to its habitat; and to check for the presence of bacteria in the gills using scanning electron microscopy.

MATERIALS AND METHODS

Habitat and Collection

Surveys and interviews were conducted from 1995 to1997 in Panay, Guimaras, and Negros islands in central Philippines (Fig. 1) to determine sources and habitat of *A. edentula*. The most accessible area to the laboratory, Brgy. San Roque in Estancia, Iloilo, central Philippines (11°14′N, 123°8′E), was chosen as the field study site and collection site of clams for laboratory studies. The area has been described in Lebata (2000).

A. edentula was collected during the ebb of a spring tide during the day with the help of a clam collector who can recognize the opening of the clams' anterior inhalant tubes (Fig. 1) on the surface of the substrate. A specialized gear made of flattened iron attached to a wooden handle was used in digging the substrate. The tube that led to the clam was then carefully traced. During collection, depth occupied by the clam was measured from the surface of the substrate, and distance from the mangrove forest was noted.

Physico-chemical parameters were monitored *in situ* at 30 cm deep. Temperature was measured using a field mercury thermometer; salinity with an Atago refractometer; pH with WTW pH 192

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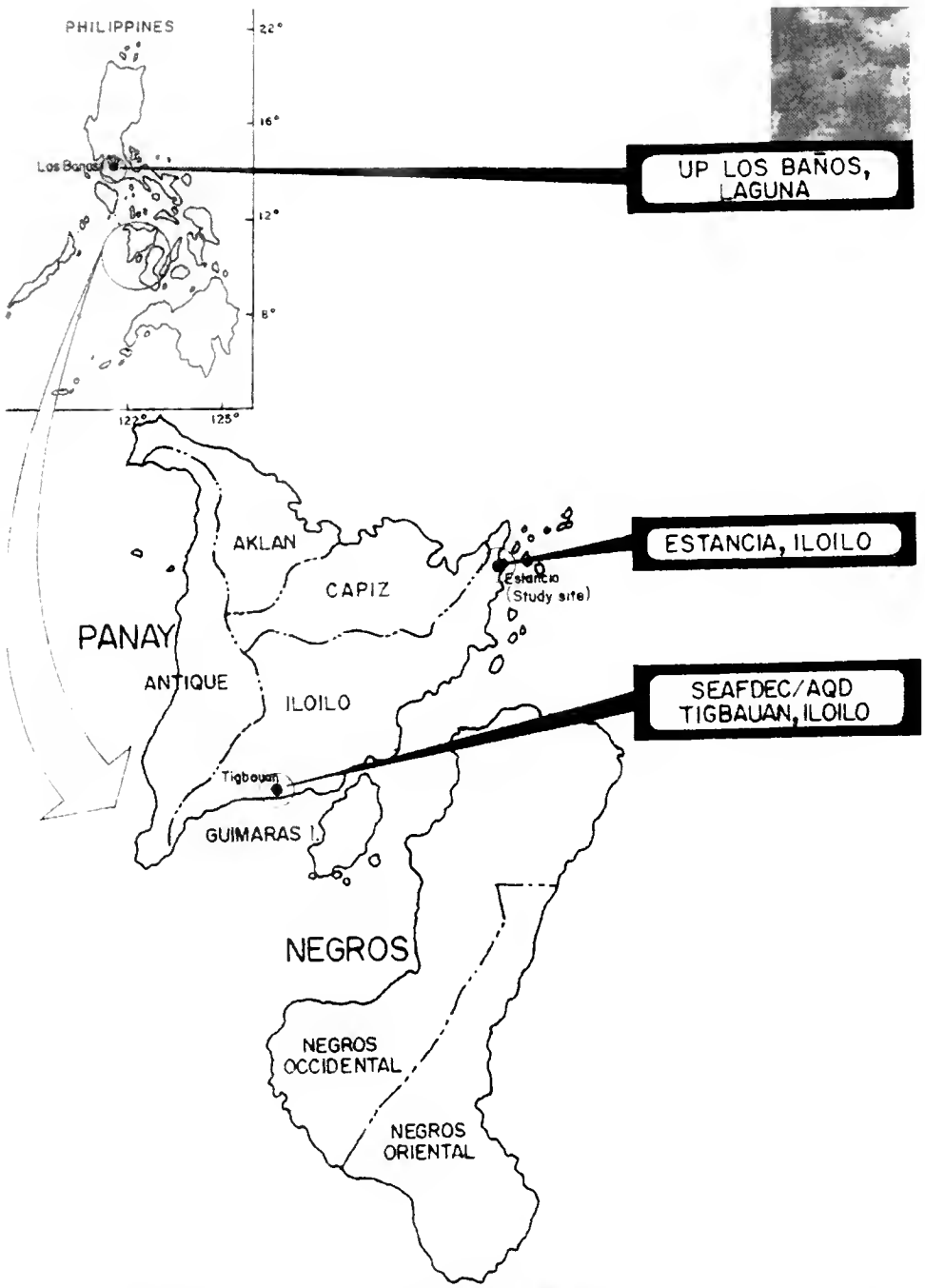

Figure 1. Map of the Philippines showing Panay, Guimaras and Negros islands surveyed during site selection; collection site chosen was Brgy. San Roque in Estancia, Iloilo, central Philippines. Inset shows the opening of *Anodontia edentula*'s inhalant siphon as seen on the surface of the substrate.

meter; D.O. with a YSI Model 51B dissolved oxygen meter; and sulfide with a Lovibond photometer PC 22.

For laboratory experiments, collected clams were transported live to SEAFDEC Aquaculture Department in Tigbauan, Iloilo, approximately 160 km from the collection site. During transport, they were placed in plastic baskets cushioned with papers soaked in seawater. Upon arrival in the laboratory, they were placed in 1-ton fiberglass tanks provided with 30–50 cm deep newly collected mangrove mud and seawater (32–35 ppt). Clams were harvested from the tanks as needed.

Anatomy

Several clams were dissected and the gross anatomy studied following Allen (1958). They were then examined for the presence of gut using an Olympus SZ-ST dissecting microscope. This was done because absence or reduction of the digestive system is a common condition in most clams containing endosymbionts in the gills (Giere 1985). Histological sections of the gonad were also made and observed under an Olympus BH2 compound microscope. Live clams were also placed in a glass aquarium provided

with seawater but no substrate to observe the extension and retraction of the foot.

Scanning electron microscopy

Newly collected live clams ranging from 50.5–62.6 mm shell length were transported to the National Institutes of Biotechnology and Applied Microbiology (BIOTECH) at the University of the Philippines, Los Baños, College, Laguna in northern Philippines (Fig. 1) on 13 May 1998 for scanning electron microscopy (SEM). Upon arrival, the biggest clam (62.6 mm SL) was dissected and the gills processed for SEM. The specimens were fixed in 3% gluteraldehyde with 0.1 M cacodylate buffer at ph 6.8 for 3 h, washed with 0.1 M cacodylate buffer 3 times at 15 min each, post-fixed in 1% osmium tetroxide for 1 h, rewashed with 0.1 M cacodylate buffer, dehydrated in graded ethanol series, and substituted with isoamyl acetate. Then they were dried in the Hitachi HCP-2 critical point dryer, mounted on supports, coated with gold and palladium using the Hitachi E101 ion sputter, and viewed under the Hitachi S-500 scanning electron microscope. Photomicrographs of the specimens were taken and compared with scanning electron micrographs of bacteria from other endosymbiont-containing invertebrates (Karl et al. 1980, Fiala-Medioni & Metivier 1986, Fiala-Medioni et al. 1986) and lucinid clams (Berg and Alatalo 1984).

RESULTS

Habitat

A. edentula thrive in the muddy substrate of mangrove areas or the adjacent mudflats. The closer the area to the mangroves the more clams can be harvested (within 20 m from the edge of the forest) and the number of clams collected decreased as collection moved seaward. Previous and recent surveys showed that they are marine and can be found nowhere in rivers or estuaries.

Clams were deeply buried in the mud ranging from 20–60 cm below the surface, but mostly in the deeper portions. Other non-lucinid bivalves inhabiting the area did not bury deeper than 15–20 cm.

The temperature ranged from 27–31°C (mean = 28.7 ± 0.28), the salinity from 32–36 ppt (mean = 34.5 ± 0.31), pH from 5.15–6.55 (mean = 5.95 ± 0.10), dissolved oxygen from 0.2–1.0 ppm (mean = 0.3 ± 0.05), and sulfide 1.5–30.0 μM (mean = 25 ± 1.78).

Anatomy

A. edentula is rounded and globose with an inflated, thin, brittle shell having irregular concentric growth lines. The shells are equivalve and the umbones are pointing anteriorly. Removing either valve, the mantle is first exposed, and removing further the mantle, the thick, fleshy, deep purple to blackish brown gills are exposed. The gills have right and left demibranchs which cover the posterior two-thirds of the gonad. On the ventral side of the latter, a little behind the posterior half, the relaxed/retracted foot is attached (Fig. 2a). So the gonad is the core of the clam, being partially covered on both sides by the gills, which are both fully hidden under the mantle. These three, plus the foot are the most prominent organs in the clam.

The foot of the clam, as observed in aquaria without mud, can extend up to 15–20 cm long and can expand to 0.8 cm in diameter (Fig. 2b). It is capable of moving around, creeping on the aquarium surface or moving up to the maximum distance its tip can reach in the water column.

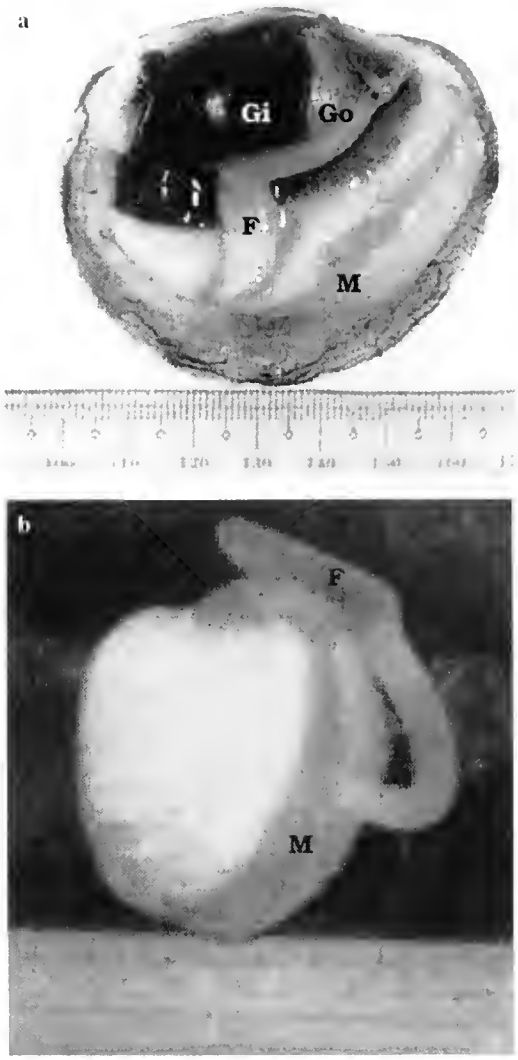

Figure 2. (a) Anatomy of a newly dissected *Anodontia edentula* showing its major organs (M: mantle, Gi: gills, F: foot, Go: gonad) after removing the right valve and the right mantle. Note the size of the relaxed foot. (b) Extended foot of live *Anodontia edentula* as seen inside a glass aquarium provided with seawater but no mud.

Dissection showed a very simple gut. The mouth enters as a slit on the anterior end of the gonad. The intestine coils inside the gonad and passes out dorsally into the rectum which lies above the dorsal side of the mantle and out to the body cavity. Efforts were made to remove the whole gut separately from the other tissues, especially the digestive structure. However, all the animals examined had developing or mature gametes. This hindered the process of tracking the very minute intestines (the biggest being 1 mm dia.) inside the gonads (Fig. 3). Unlike in other clams where the stomach is a prominent structure, no distinguishable stomach was observed in this species. Histological sections of juvenile and adult clams similarly revealed the absence of a well-defined stomach structure and the coiling inside the gonad of very small (less than 1 mm dia) intestines (Fig. 3).

Scanning electron microscopy

Scanning electron micrographs showed that bacteria in bacteriocytes (Fig. 4c) were spherical with a dia. of 8–11 μm.

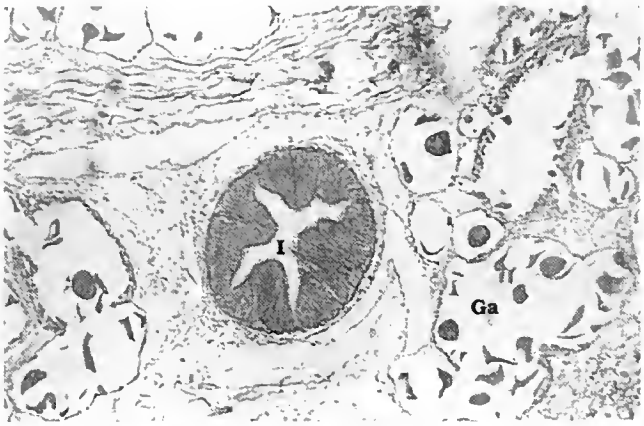

Figure 3. Cross section of *Anodontia edentula*'s intestines inside its gonad (I: intestine, Ga: gametes).

The anatomy of lucinid gills described by Distel and Felbeck (1987) may be representative for all members of family Lucinidae, and also best describes the gill anatomy of this species. Each of the two demibranches (Fig. 4a) is composed of inner and outer lamellae separated by interlamellar space. Each lamella is divided into three zones—the ctenidial filament zone, transition zone, and bacteriocyte zone. The ctenidial filament zone is the outermost zone and composed of ctenidial filaments (Fig. 4b). The innermost bacteriocyte zone (Fig. 4c) housing the bacteria lines the interlamellar space of the inner and outer lamellae. Each bacteriocyte is separated by intermediate cells devoid of bacteria (Fig. 4c).

DISCUSSION AND CONCLUSION

Surveys and salinity readings (34.5 ± 0.31 ppt) showed that *A. edentula* prefer the marine environment. They live in slightly acidic (5.95 ± 0.10) substrate which is quite anoxic. The habitat is sulfide-rich at 25 ± 1.78 μM (or 22.5 μM H_2S at pH 6.0). Sulfide measurements were higher compared to concentrations (7 ± 14.0 μM) obtained by Bagarinao and Lantin-Olaguer (1998) from 5 cm deep mud in a mangrove estuary in Guimaras Island. These concentrations are inhospitable to other aquatic non-endosymbiotic organisms as shown in the review paper of Bagarinao (1992). However, for endosymbiotic clams this may be ideal. According to Distel and Felbeck (1987), the clams should be strategically situated in an interface between a sulfide-generating zone (anoxic) and water with sufficient oxygen (oxic). The location of *A. edentula* (20–60 cm deep in mangrove mud) may allow it direct access to sulfide, and the inhalant tube provides access to oxygenated water; both sulfide and oxygen are essential for its survival.

The anatomy of the clam further supports endosymbiosis. The foot of *A. edentula* can extend up to several times longer than its shell length. Just like other lucinids, this gives it the capacity to construct a ventilation burrow (Reid & Brand 1986) and allows it to draw water from the surface (Dando et al. 1985). The tip of the foot is specialized for the construction of this inhalant tube. It is provided for with glands that lay down mucus for the building of the tube (Allen 1958). In cases where sulfide is limiting, Childress et al. (1991) have proven that the foot can dig deeper and is responsible for the uptake of sulfide from deeper parts of the substrate. Sulfide is taken up across the foot of the clam and into the blood that transports it to the gills. The host is therefore responsible for delivering the sulfide to the bacterial symbiont.

Sulfide is a highly reduced energy molecule, and a variety of biological systems have evolved to oxidize sulfide in orderly enzyme-regulated steps to harness the energy and avoid poisoning (Bagarinao 1992). In a clam-bacteria symbiosis, bacteria are assumed to provide the mollusk with chemosynthetically fixed carbon dioxide via aerobic oxidation of sulfide (Vetter 1985). Oxidation of sulfides and other reduced sulfur compounds provides

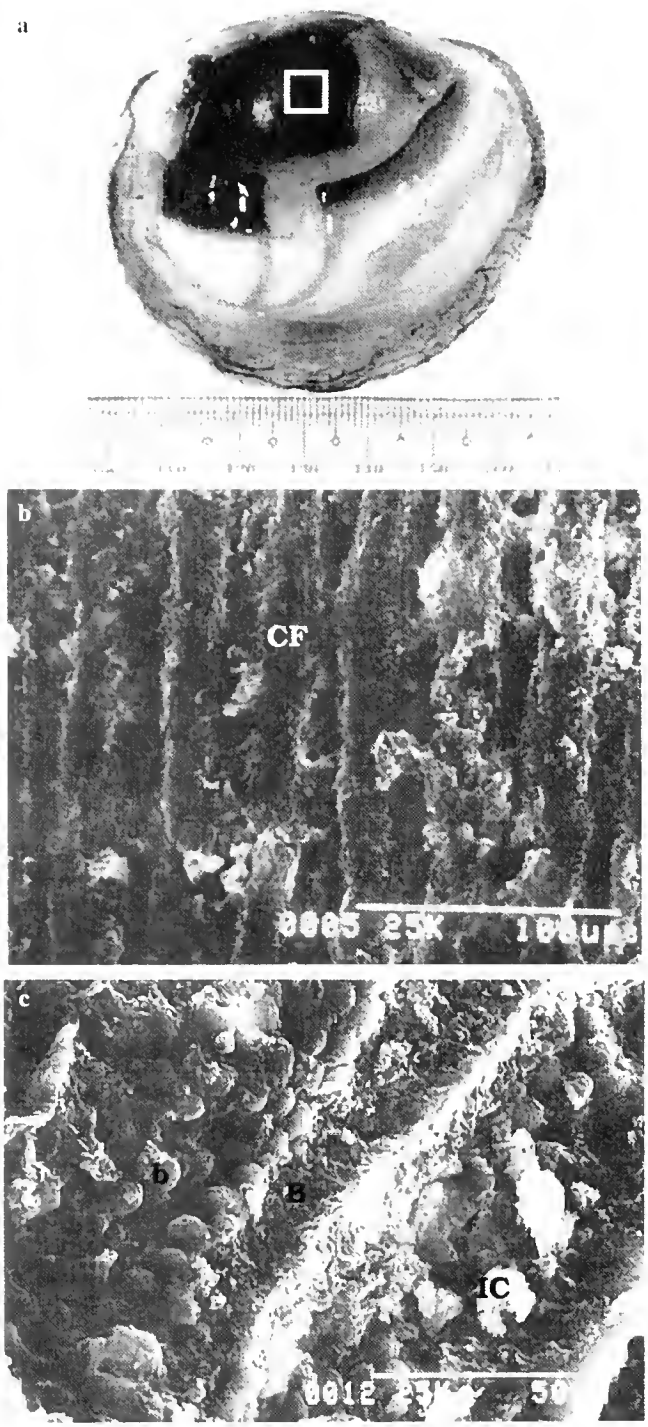

Figure 4. Gill structure of *Anodontia edentula* taken under scanning electron microscope. (a) Portion of the gill's right demibranch (boxed) cut for SEM. (b) Scanning electron micrograph of the boxed region showing the ctenidial filaments (CF). (c) Boxed region rotated 90° about its horizontal axis, exposing the bacteriocytes (B) housing the spherical endosymbiotic bacteria (b). Note also the presence of intermediate cells (IC) devoid of bacteria.

energy to the bacteria to fix carbon dioxide into organic compounds which become available to the host clam (Distel & Felbeck 1987, Kelly & Harrison 1989). The Calvin cycle is the main metabolic pathway used by the bacteria to convert carbon dioxide to organic carbohydrates powered by the energy (ATP) generated from the oxidation of sulfides (Distel 1998). In the process of oxidation, sulfide is converted into elemental sulfur and stored for future use (Childress & Mickel 1982, Vetter 1985, Distel 1998).

Dissection and histological examination revealed a very simple gut (Fig. 3), similar to that observed for other lucinid species, *Codakia orbicularis* (Berg & Alatalo 1984), and *Parvilucina tenuisculpta* (Reid & Brand 1986). This condition also parallels the observation of Allen (1958) in several genera (*Codakia, Divaricella, Loripes, Lucina, Myrtea* and *Phacoides*) belonging to family Lucinidae.

The stomach and some parts of the intestines outside the gonad were observed in some members of the families Pectinidae, Ostreidae, Pinnidae and Corbiculidae examined by Okutani et al. (1985). Ruppert and Barnes (1994) also described the clear digestive diverticula and stomach outside the gonads in *Pecten* and other filter feeders. The well-defined digestive systems in these families contrasted the very simple and reduced digestive structures in *A. edentula* and other lucinid species.

According to Giere (1985), gut reduction is a common condition in most clams containing endosymbionts in the gills. The great reduction or absence of a digestive system in the host species indicates that bacterial chemosynthesis is vital to the host's nutritional scheme (Distel & Felbeck 1987, Distel 1998). Hence, gut reduction in *A. edentula* is further evidence of an endosymbiotic relationship.

Furthermore, the gills of *A. edentula*, described to be thick, fleshy and deep purple to blackish brown in color, characterize the gills of endosymbiotic clams which were noted for being thick and opaque, contrasting sharply the thin, delicate, translucent gills of most bivalves (Distel 1998). This agrees with the description of Allen (1958) for several genera of lucinids, Dando et al. (1985) for *Myrtea spinifera*, and Distel and Felbeck (1987) for *Lucinoma aequizonata, Lucinoma annulata*, and *Lucina floridana*, all of which are known for endosymbionts. Similarly, Felbeck et al. (1981) observed the same gill coloration in bivalves collected from sulfide-rich habitats containing sulfide oxidation enzymes in contrast with the small light-colored gills of those lacking these enzymes. According to Distel and Felbeck (1987), the dark coloration and thickness of the gills are due to the presence of a thick layer of subfilamentar tissue perforated by regular arrays of bacteriocyte channels formed by bacteriocyte cylinders containing the bacterial symbionts. The nearly black appearance of the gills may also be attributed to cytoplasmic hemoglobin (Kraus & Wittenberg 1990). Although it is relatively uncommon in symbiont-free gills, cytoplasmic hemoglobin is a nearly constant feature in symbiont-harboring gills of bivalves and may function in the delivery of oxygen and sulfide to ensure symbiont autotrophy and host cell respiration (Kraus 1995).

Scanning electron photomicrographs showed only one type of bacteria in the bacteriocyte zone of the gills (Fig. 4c). The bacteria were coccoid and almost of uniform sizes. According to Dando et al. (1985) and Conway et al. (1992), only one type of endosymbiont exists in every clam host, and that bacterial pleomorphism may only represent varying developmental stages of a single species of symbiont.

The absence of bacteria in the mantle and foot and their confinement in the gills may be explained by the role of the gills in gas exchange. The bacteria must therefore thrive in the gills to ensure access to oxygen and sulfide obtained from the substrate. Doeller et al. (1988) isolated two different hemoglobins occurring in nearly equal concentrations in the gills of *Solemya velum*. The sulfide reactive hemoglobin delivers sulfide to the bacteria and the non-sulfide reactive one delivers oxygen. Aside from their role in gas exchange, the presence of bacteriocytes (which are absent in the mantle and foot) made the gills suitable for bacterial settlement.

Scanning electron micrographs showed that the morphological appearance of bacteria in gill samples resembles those observed by Berg and Alatalo (1984) from the gills of a lucinid, *C. orbicularis*, and in other endosymbiont-containing invertebrates (Karl et al. 1980, Cavanaugh et al. 1981, Fiala-Medioni & Metivier 1986, Fiala-Medioni et al. 1986). The measured size of the bacteria (8–11 μm) is in the same range as those observed for *S. velum* (2–10 μm: Cavanaugh 1983), *Anodontia philippiana, Lucina multilineata, Lucina costata* and *Lucina radians* (maximum of 8.9 μm: Giere 1985), *L. aequizonata* and *L. annulata* (5–10 μm: Distel & Felbeck 1987), and *Solemya borealis* (3–15 μm: Conway et al. 1992). The presence of intermediate cells separating bacteriocytes was also noted by Giere (1985) in 4 species of lucinids collected from Bermuda. The bacteria and the gill structure observed under SEM agree with observations of other papers as described by the literature in previous paragraphs and may be strong supporting evidence to prove clam-bacteria endosymbiosis in *A. edentula*.

As supported by its habitat, anatomy, and the bacteria in its gills, we cannot consider *A. edentula* to be an ordinary mangrove clam. Its endosymbiotic capacity should be considered in future studies, whether on its biology or culture.

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SHELL MORPHOGENESIS OF SEVERAL VENERID BIVALVES

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ABSTRACT Morphogenetic series of venerids *Protothaca euglypta*, *Ruditapes philippinarum*, and *Callista brevisiphonata* were constructed to determine the morphological criteria for identification of their larvae and juveniles from 0.2 up to 1.5–2.0 mm shell length. Larvae, post-larvae, and juveniles were reared in the laboratory and collected from plankton and benthic samples. The important distinguishing characters (shell outlines, provincular denticles, sequence of cardinal teeth formation, recapitulative sculpture, and others) of venerid bivalves in different stages of development are presented. Morphogenetic series can provide the basis for the phylogenetic reconstruction and be a successful aid in solving the problems of identification and ecology of bivalves in early stages of their ontogeny.

KEY WORDS: veneridae, *Protothaca*, *Ruditapes*, *Callista*, morphogenesis, hinge, larval-juvenile morphological features

INTRODUCTION

Venerids are a common group of bivalves in the northwestern part of the Sea of Japan. There are 10 venerid species in this area. The taxonomic differences, vertical and spatial distribution patterns, density, and reproductive ecology of these clams are relatively well studied (Habe 1951, Habe 1977, Scarlato 1981, Ponurovsky 1993, Sato 1999). However, these studies are largely concerned with adult animals. Little information is available on the pelagic stages of some taxa and juveniles (Kulikova & Kolotukhina 1989), which considerably differ from adults in their morphology. A lack of efficient criteria by which to discriminate among venerid bivalves in early juveniles (~0.3–3 mm shell length) hinders the studies of their recruitment both in natural populations and on artificial substrata. At the same time, almost all of these clams are objects for harvesting and, potentially, cultivation in the Sea of Japan. The purpose of our study is to determine the morphological criteria for the identification of three common venerid species in early stages of their ontogeny.

MATERIALS AND METHODS

Adult *Protothaca euglypta* (Sowerby, 1914), *Ruditapes philippinarum* (Adams et Reeve, 1848), and *Callista brevisiphonata* (Carpenter, 1865) from the northwest Sea of Japan, their larvae, post-larvae, and juveniles were used in this study. Adults and juveniles were sampled by SCUBA-diving in different biotopes down to 20 m depth. Larvae of *R. philippinarum* and *C. brevisiphonata* were collected on a 96-μm nylon plankton net from a small boat. Larvae and juveniles of *P. euglypta* were reared in the laboratory and picked out from benthic samples. Laboratory specimens were kept using the appropriate methods (Loosanoff & Davis 1963) and fed with *Isochrysis galbana*, *Chaetoceras muelleri*, and *Dunaliella salina*.

Larval and juvenile shells were cleaned in a 5–10% solution of sodium hypochlorite, disarticulated with a fine needle, rinsed with distilled water, and embedded in glycerin jelly for light microscopy. Morphogenetic series of two types were constructed. Progressive series consisted of larval stages up to the juvenile and

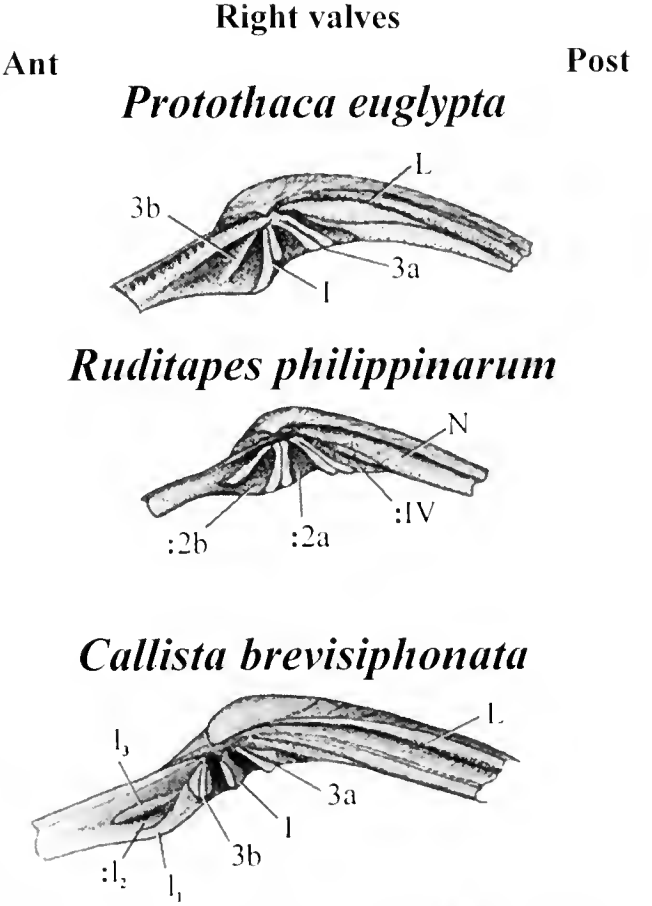

Figure 1. Hinge-tooth nomenclature of venerid shells (after Bernard, 1895 and Le Pennec, 1980, with alterations). Roman numerals, Arabic numerals followed by a lower case letter, and letters followed by subscript numerals indicate developing hinge teeth. I, the first tooth of the right valve; :IV, pit for the second tooth of the left valve. 3a, the initial tooth of tooth III of the right valve; 3b, the secondary tooth of tooth III; :2a and :2b, pits for the initial and secondary teeth of tooth II of the left valve. I_1, the first lateral tooth of the right valve; I_3, the second lateral tooth of the right valve; $:I_2$, pit for lateral tooth of the left valve. Ant, anterior; Post, posterior; L, ligament; N, nymph.

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adult stages, and retrospective series consisted of adults to juveniles including early post-larval stages. A system of hinge-tooth notation used in this study (Fig.1) is based both on Bernard's (1895) nomenclature and comments of Le Pennec (1980).

RESULTS

The examined distinguishing morphological features of three venerid species are summarized in Table 1.

A series of *P. euglypta* (Fig. 2) begins with pelagic larvae of 200 μm shell length. The larval shell is almost regularly rounded and equilateral. The umbones are high and the distinct ligament pit is posteriorly situated. There are 7–8 well-developed rectangular provincular denticles that elongate as the shell grows.

At a shell length of 210–260 μm cardinal teeth form. At first, tooth II appears as an oval knob on the narrow and elongate plateau in the antero-dorsal part of the left valve. On the right valve, cardinal tooth I forms at a shell length of more than 240–260 μm. Similar to tooth II, tooth I is placed on the dental plateau in the anterior part of the valve. Initially, this tooth is rounded.

At a shell length of 250–400 μm, the anterior end of the shell is longer than the posterior end and the umbones are higher than in the previous stage. The number of provincular denticles remains nearly unchanged, but their shape becomes more ventrally elongate. The dental plateau of tooth II of the left valve is broadened and elongated, and the new bulge develops in the antero-ventral part of tooth II. This bulge develops into a high knob that is separated from tooth II by a marked saddle. Tooth II acquires a bicuspidate shape.

On the right valve, tooth I becomes high and its shape changes to oval. Its plateau is broadened posteriorly and ventrally. The new dental plateau of cardinal tooth III develops on the surface of first plateau between tooth I and the anterior end of the provinculum. The outer shell surface bears 2–3 concentric ridges that are more distinct at the posterior field of the shell.

At a shell length of 400–600 μm, the provincular denticles and ligamental pit are overgrown by the plateau of teeth I and III. At the outer posterior end of the shell, 1–2 low concentric ridges appear as the initial stage of the separation of the escutcheon. The lunule formation may also occur in this stage of development.

At a shell length of 600–900 μm, a new bulge develops along the dorsal margin of the dental plateau of tooth III. This bulge is elongated and connected with the base of tooth III. Tooth III consists of two parts: the ventral part 3a and the anterior part 3b. At the ligamental pit, a new broad tooth pit appears that is anteriorly limited by tooth 3a. The posterior and dorsal parts of this pit are occupied by an internal ligamental layer (resilium). The anterior part of the pit is intended for tooth IV of the left valve. The posterior end of the shell becomes thicker, and the formation of the escutcheon takes place there. The outer shell surface bears several high concentric ridges. In addition to these ridges the radial ribs develop, and the formation of the external ligament is completed at 900–1500 μm shell length.

The series of *R. philippinarum* (Fig. 3) begins at the larval stage of 230 μm shell length. The provicultum bears 12–14 small irregular denticles. The shell is inequilateral; the anterior end of the shell is almost straight and slightly longer than the posterior end. The umbones are broad and low, and the distinct ligament pit is posteriorly located.

At a shell length of 250–350 μm, the dental plateaus of cardinal teeth I and II begin to form. Tooth II appears first, and when the shell length is approximately 300 μm, it is clearly seen as an oval and high knob at the margin of its dental plateau. On the right valve, dental plateaus of teeth I and III develop at this stage. The

TABLE 1.

Morphological features of larval and juvenile venerid bivalves (for the right valves).

Stage	Species		
	Protothaca Euglypta	*Ruditapes Philippinarum*	*Callista Brevisiphonata*
180–300 μm	Shell high and equilateral, with 7–8 regular denticles; ligament pit distinct; outer surface with growth lines.	Shell inequilateral, with 12–14 small irregular denticles; ligament pit distinct; outer surface covered with concentric striation.	Shell oval, slightly inequilateral, without any denticles; ligament pit indistinct; outer surface with fine and irregular concentric grooves.
250–350 μm	Formation of cardinal tooth I; outer surface covered with concentric growth lines.	Formation of dental plateau for cardinal teeth I and III; outer surface with weakly waved concentric and regular grooves.	Formation of cardinal tooth III; overgrowing of ligament pit; formation of dental plateau for cardinal tooth I; outer surface with nearly regular concentric grooves.
350–600 μm	Formation of cardinal tooth III; overgrowing of ligament pit; outer surface with widely spaced concentric ridges.	Formation of cardinal tooth I; overgrowing of ligament pit; outer surface with regular concentric grooves and widely spaced radial striae.	Formation of cardinal tooth I; bifurcation of tooth III into 3a and 3b; formation of external ligament; outer surface with nearly regular concentric grooves.
600–900 μm	Bifurcation of tooth III into 3a and 3b; formation of ligament-and-tooth pit :IV; formation of escutcheon; outer surface with concentric ridges.	Formation of cardinal tooth III and ligament-and-tooth pit :IV; outer surface with concentric grooves and radial ribs.	Formation of lateral tooth I_1.
900–1,500 μm	Formation of external ligament; outer surface with concentric ridges and radial ribs.	Bifurcation of tooth III into 3a and 3b; formation of external ligament.	Formation of lateral tooth I_3.

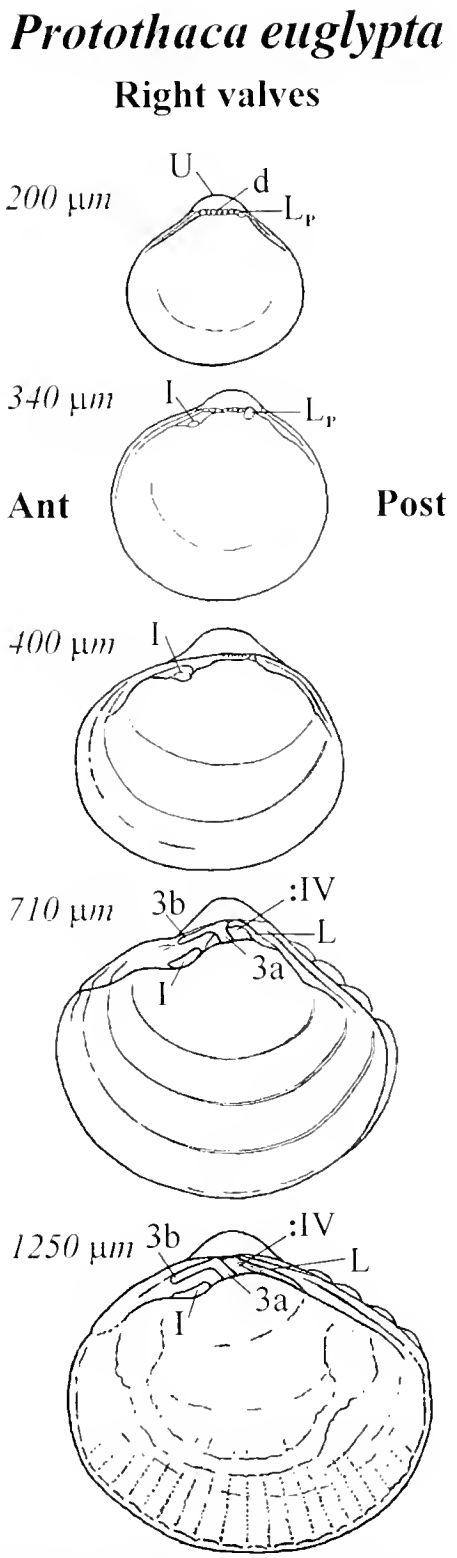

Figure 2. Morphogenetic series of *Protothaca euglypta*, the Sea of Japan. Abbreviations and numerals are the same as given in Figure 1. d, provincular denticles; L_p, ligament pit; U, umbo. Shell lengths are given.

outer shell surface is covered with weakly waved concentric and regular grooves.

At a shell length of 350–600 μm, cardinal tooth I develops. The internal layer of the shell appears at this stage. At the next stage (600–900 μm), cardinal teeth III and IV appear. The shell surface has both concentric grooves and radial ribs. There are approximately 28–32 ribs.

At 900–1,500 μm, the shell outlines become more anteroposteriorly elongate. Teeth II and III bifurcate into 2a and 2b and 3a and 3b, respectively. On the posterior side of the ligament-and-tooth pit :IV the external ligament develops. On the internal layer of shell, the scars of anterior and posterior adductors and the pallial line appear. The number of distinct radial ribs of shell surface varies from 30–32 to 38–42. In the first case, the ribs are flattened, low, and smooth or weakly waved. Near the shell margin the ribs are 40–60 μm wide; the width of the intercostal grooves does not exceed 10–15 μm. In the latter case, the ribs are high, broadly spaced (20–30 μm), and have nodular tops. In both cases, rare concentric bridges may appear in the posterior and sometimes in the central area of the shell. The distance between these bridges is about 110–140 μm.

Larval shells of *C. brevisiphonata* 220–240 μm long (Fig. 4) are devoid of any provincular denticles. The shell is oval and slightly inequilateral. The umbones are low and the ligament pit is indistinct. The shell surface looks smooth and lustrous. The formation of the cardinal teeth begins at a shell length of 250–350 μm with the appearance of tooth II on the left valve. Then, tooth III and the dental plateau for tooth I appear on the right valve. Tooth I is the last to form at this stage. The outer surface is covered with nearly regular concentric grooves.

At a shell length of 400–600 μm, tooth III bifurcates into 3a and 3b teeth. The external ligament begins to develop on the posterior side of pit :IV, and its formation is completed when shell length exceeds 800–900 μm. The escutcheon and lunule are indistinct.

At a shell length of 600–900 μm, the first lateral tooth appears, and the second one forms at 900–1,000 μm shell length. The formation of lateral teeth ceases at a shell length exceeding 1,500 μm. The new internal shell layer appears at a shell length of more than 1,000 μm.

DISCUSSION

There are 10 venerid species in the northwestern part of the Sea of Japan (Fig. 5). Adult forms of these taxa have distinguishing morphological features (Scarlato 1981). The constructed morphogenetic series are representative of the clearly distinctive characteristics of larval and juvenile *P. euglypta*, *R. philippinarum*, and *C. brevisiphonata*. Morphogenetic series of other venerid species are not complete. Some of the reasons for this include the comparative rareness of larval and post-larval stages in plankton and benthic samples and difficulties with spawning of adult venerids and rearing of their larvae in the laboratory. Nevertheless, a number of available shells would be sufficient to identify these species. For example, juvenile *Mercenaria stimpsoni* (Gould, 1861) has a wide dental plateau of tooth III, the sculpture with ridges and the comparatively thickened shell on which this species differs from other venerids of the Sea of Japan. Juveniles of *Saxidomus purpuratus* (Sowerby, 1855) possess lateral teeth but differ from *C. brevisiphonata* in their sculpture. The shell outlines of *Liocyma fluctuosa* (Gould, 1841) at a shell length less than 1,000–1,200 μm are close to those of *C. brevisiphonata*; however, the shell surface of *L. fluctuosa* is smooth and lustrous, and lateral teeth are lacking.

The most noticeable differences among adult *P. euglypta*, *Protothaca jedoensis* (Lischke, 1874), and *Callithaca adamsi* (Reeve,

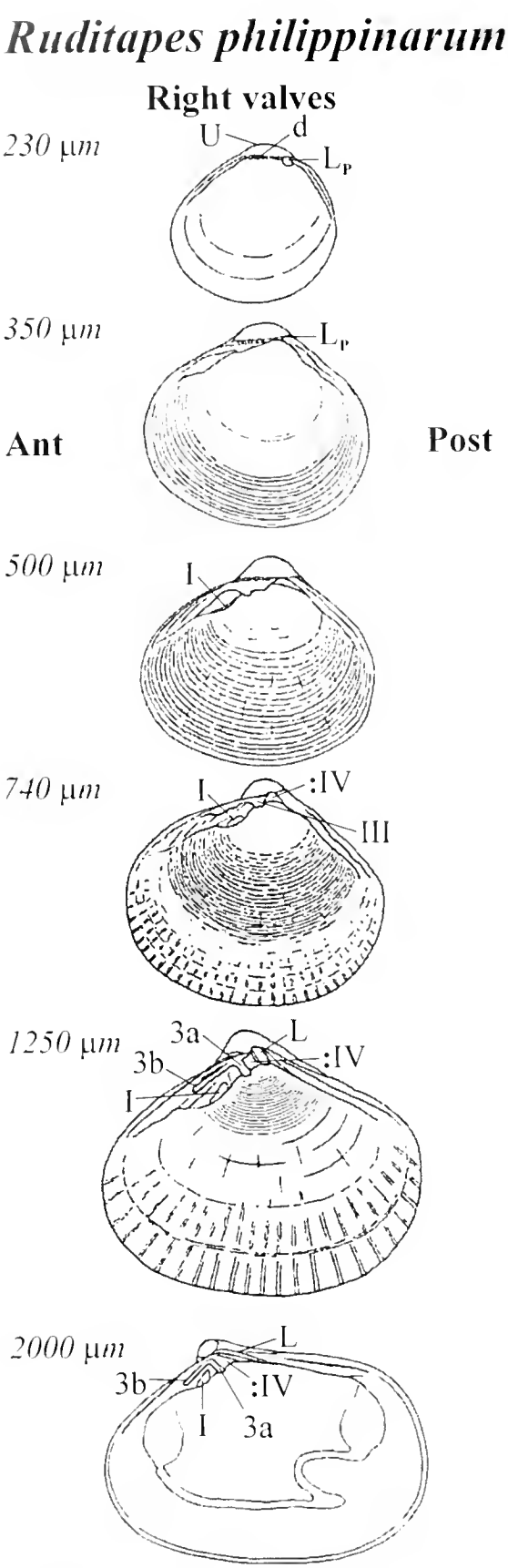

Figure 3. Morphogenetic series of *Ruditapes philippinarum*, the Sea of Japan. Abbreviations and numerals are the same as given in Figures 1 and 2. III, the second tooth of the right valve. Shell lengths are given.

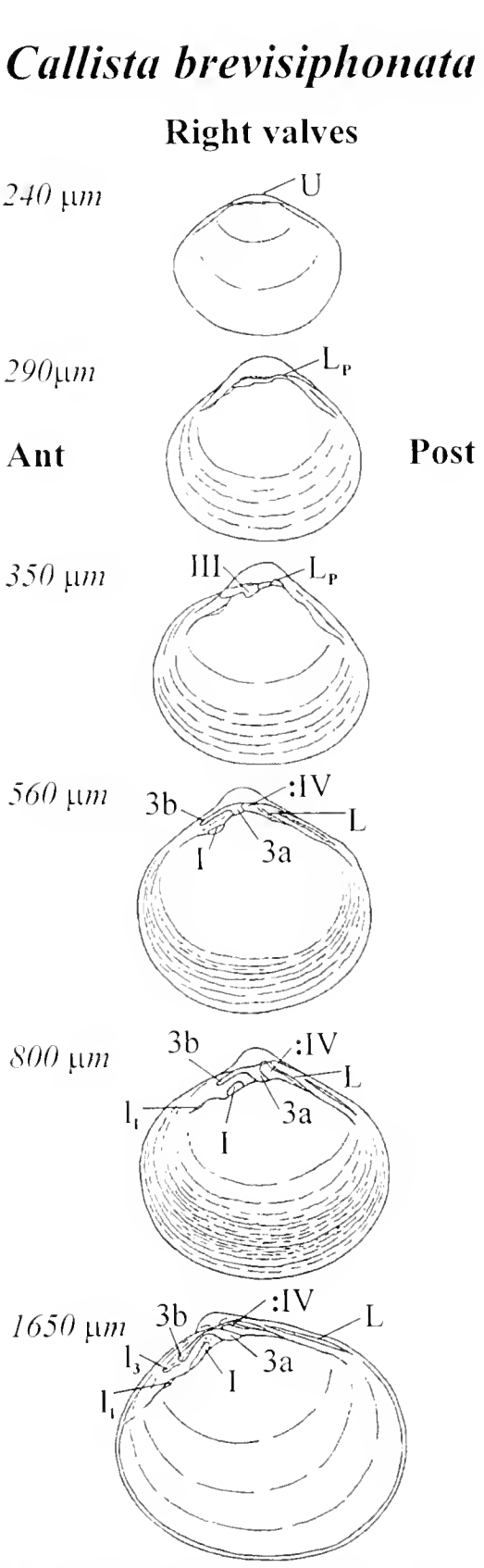

Figure 4. Morphogenetic series of *Callista brevisiphonata*, the Sea of Japan. Abbreviations and numerals are the same as given in Figures 1, 2, and 3. Shell lengths are given.

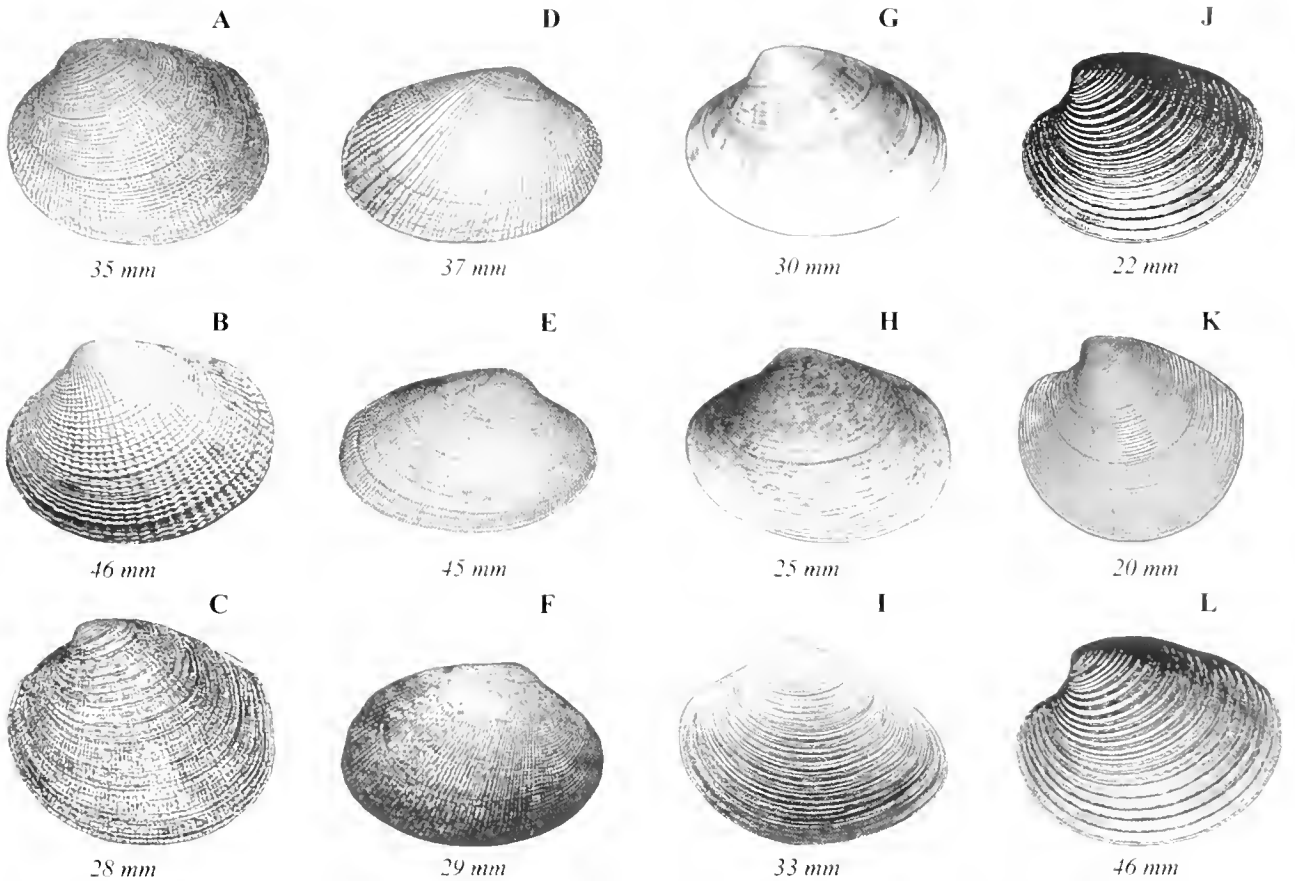

Figure 5. Venerid species of the Sea of Japan and venerids from the Sea of Okhotsk and the South China Sea, given for comparison: (A) *Protothaca euglypta* (Sowerby, 1914), Krasnaya Skala Bight, the Sea of Japan; (B) *Protothaca jedoensis* (Lischke, 1874), Vostok Bay, the Sea of Japan; (C) *Callithaca adamsi* (Reeve, 1863), Vladimir Bay, the Sea of Japan; (D) *Ruditapes philippinarum* (Adams and Reeve, 1848), Krasnaya Skala Bight, the Sea of Japan; (E) *Ruditapes philippinarum* (Adams and Reeve, 1848), the southern coast of Kunashir Island, the Sea of Okhotsk; (F) *Ruditapes philippinarum* (Adams and Reeve, 1848), Ho-Man Island, the South China Sea; (G) *Callista brevisiphonata* (Carpenter, 1865), Sivuchjya Bight, the Sea of Japan; (H) *Saxidomus purpuratus* (Sowerby, 1855), Amursky Bay, the Sea of Japan; (I), *Liocyma fluctuosa* (Gould, 1841), Uspeniya Bight, the Sea of Japan; (J) *Mercenaria stimpsoni* (Gould, 1861), Pogranichnaya Bight, the Sea of Japan; (K) *Phacosoma japonica* (Reeve, 1850), Sivuchjya Bight, the Sea of Japan; (L) *Dosinella angulosa* (Philippi, 1847), Ussury Bay, the Sea of Japan. Shell lengths are given.

1863) (Fig. 5, A–C) concern the shell outlines and sculpture (Scarlato 1981). At the same time, these features are retraced up to 3–5 mm shell length at the retrospective morphogenetic series. The outlines of smaller juvenile shells vary to a considerable extent, but their sculpture, which is the characteristic of specific rank, is comparatively uniform. In this case, the additional information concerning geographical distribution, nature of biotope, periods of spawning and settlement can be useful for the taxonomic differentiation of juvenile stages of *P. euglypta*, *P. jedoensis*, and *C. adamsi*.

A peculiarity of venerid species of the Sea of Japan is that they all are classified into different genera except for two species of genus *Protothaca* (Kafanov 1991). Because of this, the specific features of adult venerids often reveal the characteristics of a higher rank, generic or familial, including shell outlines, availability or lack of marginal crenulation, radial or concentric ribs, and other characteristics (Scarlato 1981, Harte 1998). At the same time, the outlines and height of cross-sections of the ribs and size of intercostal spaces are usually considered as specific characteristics (Tchang et al. 1960, Zhuang 1964, Matsukuma 1986). In connection with these differences, it is often difficult to determine the taxonomic rank of juveniles and to compare them with taxa from other areas. For example, the comparison of post-larval stages of *M. stimpsoni* from the Sea of Japan with Atlantic species *M. mercenaria* (Goodsell et al. 1992) shows that shell outlines may be of a generic rank. However, taking into account the availability of subspecies of *M. mercenaria*, this characteristic might be considered intraspecific.

Similarly, the introduced Atlantic species *R. philippinarum* considerably differs from *Ruditapes decussata* in the morphology of the juvenile hinge (Le Pennec 1980). Unfortunately, the shell sculpture, which is one of the principal features of specific rank, was not considered in that study. At the same time, juvenile *R. philippinarum* from the Sea of Japan have both the recapitulative concentric sculpture and the sculpture of adults (Fig. 3). The former develops at the early post-larval stage and is an important taxonomic characteristic of probably generic rank. At a shell length of 500–600 μm, the concentric sculpture changes to a radial one of two types that can also be seen in adults (Fig. 5, D and E). There are two points of view about the taxonomy of this species. In one opinion, molluscs having this different sculpture may belong to two different species (Tchang et al. 1960, Golikov & Scarlato 1967). In the other opinion (Oniwa et al. 1988), they may be of the same species, revealing the intraspecific latitudinal variability typical of the family Veneridae (Tanabe and Oba 1988, Sato 1996, Sato 1999). Taking into account the morphological differ-

ences of juvenile and adult *R. philippinarum* (Fig. 5, D–F), we are inclined to the first opinion and suggest that the taxonomy of this species inhabiting the wide area from the southern part of the Sea of Okhotsk to the waters of northern New Guinea be revised.

Based on the results of this study, construction of morphogenetic series that are representative of specific features of ontogeny and phylogeny of bivalves would appear to be useful for comparison of characteristics of larval and post-larval development of closely related species and their identification. In this case, the most important characteristics are shell outline; sequences of cardinal and lateral teeth formation; duration of teeth formation when they are not yet functional; formation and then overgrowing of such structures as recapitulative sculpture, internal ligament or ligament pit, or provincular denticles; and sequences of the formation of sculpture, external ligament, posterior–dorsal ridge and escutcheon, shell internal layer, sinus of pallial line, and pigment spots. Morphogenetic series can provide the basis for phylogenetic reconstruction and detailed analysis of the level of supergeneric taxa. Morphogenetic series make it possible to reveal the regularity of recruitment, to estimate the mortality of age cohorts in different stages of ontogeny and in different biotopes, and to solve a number of other ecological problems usually concerning only adults because of the difficulties of identification of species in early stages of ontogeny.

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THE IMPACT OF RISK INFORMATION ON THE DEMAND FOR GULF OF MEXICO AND CHESAPEAKE OYSTERS

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ABSTRACT The public, through information tied to warning labels and media attention, has been increasingly made aware of the possible health risks associated with the consumption of raw oysters harvested from the Gulf of Mexico. The purpose of this paper is to determine whether this information has reduced demand for the Gulf of Mexico product, the subject of the information, and a closely related product: oysters harvested from the Chesapeake. Results suggest that the information (i.e., warning labels and related media attention) has resulted in a reduction in demand for these products. As a result of the decline in demand, the Gulf of Mexico price was estimated to be about 45% lower than it would have been in the absence of the information. The Chesapeake price was estimated to be almost 15% below what it would otherwise have been.

KEY WORDS: *Vibrio vulnificus*, Gulf of Mexico, Chesapeake, oyster demand

INTRODUCTION

The oyster industry operates on both the East and the West Coasts of the United States. Landings from the East Coast (i.e., the Atlantic estuaries and Gulf of Mexico), dominated by the species *Crassostrea virginica* (Eastern oyster), averaged 28 million pounds annually during 1993–1997 (National Marine Fisheries Service 2000). Landings from the West Coast (i.e., the Pacific estuaries), dominated by the species *Crassostrea gigas*, averaged about 10 million pounds annually during the same period.

Vibrio vulnificus is a natural bacteria species that grows in the warmer waters of the Gulf of Mexico (hereafter referred to as the Gulf). Consumption of raw oysters contaminated with *V. vulnificus* can cause illness and death among the small segment of the population with weakened immune systems. As noted in a recent issue of the *Nutrition Action Healthletter* (Corcoran 1998): "[e]very year, more than 50 people become ill—and at least 10 die—after eating uncooked Gulf of Mexico oysters that are contaminated with *Vibrio vulnificus* bacteria." The presence of *V. vulnificus* in oysters is highly correlated with water temperature in the Gulf and increases significantly during the warmer summer months. More than 80% of the deaths attributable to the bacteria since 1978 have occurred in the six-month period ending in October, and more than 50% have occurred in the three-month period of August through October. No deaths attributable to *V. vulnificus* contamination in raw oysters have been reported during the first three months of the year (D. Park, pers. comm.).

California, in response to eight confirmed *V. vulnificus*–related cases between 1985 and 1990, initiated a program in March 1991 which required all restaurants and stores selling Gulf oysters to notify potential customers that the "consumption of raw oysters can cause illness and death among people with liver disease, chronic illness, or weakened immune systems" (Liddle 1991). California's mandatory warning received extensive press in local papers (and the trade literature) throughout the country, including the Gulf. Furthermore, about the time that California mandated warning labels, many of the Louisiana dealers began to voluntarily place warning labels on products sold in local markets (the warning label has since become mandatory in Louisiana as well as in some other Gulf states).

One can hypothesize that state actions (i.e., mandatory warning labels) and the associated media attention, to the extent that they increased consumer awareness of the health risks attached to the consumption of raw Gulf oysters, have resulted in a change in consumer preference (i.e., a reduction in demand) for Gulf oysters, particularly the raw product. One can further hypothesize that consumer preference (demand) for closely related substitutes that were not the subject of warning labels and media attention, such as oysters produced in the Chesapeake, may have been altered as a result of the increased information attached to the Gulf product.

Literature pertaining to consumer behavior with respect to food-borne contamination events was reviewed by Strand (1999), who reached the following generalizations concerning formation of perceptions by consumers from information presented to them. First, the information, which is subjectively evaluated with the process not being easily described, is critical to perception formation. Uncertainty contained in the information can also be critical in perception formation. Finally, the credibility of the information depends on the source of the information.

The formation of perceptions (or changes) can, in turn, alter consumer choice. With respect to changes in consumer choice, Strand (1999) first concluded that consumers react to negative news by reducing demand for the product and/or by taking defensive actions to lower the level of health risk. The author also concluded that as a result of uncertainty (e.g., uncertainty of the marketing channels through which they obtain their consumables), consumer choice could be altered (i.e., a reduction in demand) even though there is no scientifically supported risk to them from their normal consumption. According to Strand, there is evidence to suggest that consumers avoid uncontaminated seafood after news of contamination incidents, presumably due to lack of information. Finally, Strand suggests that changes in consumer choice (demand) for products containing persistent toxic compounds (like DDT) appear to be a reaction to cumulative news. Although the effects of this news may decay over time, the decay will be slow.

The synthesis provided by Strand (1999) yields several insights relevant to analysis of potential change in oyster demand as a result of warning labels and negative publicity. First, uncertainty is likely to be a major factor. The uncertainty is inherent in both the information presented to the consumer as well as in consumers themselves as to whether they possess the health characteristics (i.e., liver disease, chronic illness, or a weakened immune system)

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that would make the consumption of raw oysters "risky." Second, one could argue that the change in demand for Gulf oysters is analogous to Strand's discussion regarding changes in demand for food products containing persistent toxic compounds. Specifically, whereas *V. vulnificus* is not a toxic compound, like a toxic compound it is persistent in nature and continues to receive press several years after initiation of warning labels. Finally, based on Strand's review, the inability of the "average" consumer to differentiate the Gulf product from oysters harvested in other regions of the United States, particularly the Chesapeake (the other historically major producing region of the species *Crassostrea virginica*) suggests that media attention associated with the Gulf product may have adversely influenced demand for oysters harvested from outside the Gulf.

The primary objective of this paper is to determine the extent to which increased information (warning labels and publicity) has affected the demand for Gulf oysters, the subject of the information, and the demand for a closely defined substitute that is not the subject of the information. Given its close relation to the Gulf product, the Chesapeake product was selected as the substitute product to examine. An ancillary objective is to measure the impact of changes in other relevant variables (e.g., landings and income) on the demand for oysters harvested from the Gulf and Chesapeake.

MODEL SPECIFICATION

Gulf Demand Model

For purposes of analysis, the demand for Gulf oysters is specified as

$$\begin{aligned} PG_t = {} & \beta_0 + \beta_1 QG_t + \beta_2 INC_t + \beta_3 VUL_t + \beta_4 SEAS_t \\ & + \beta_5 (SEAS^* VUL)_t + \beta_6 (QG^* SEAS)_t + \beta_7 LATX_t \\ & + \beta_8 LPG_t + \epsilon_t \end{aligned} \quad (1)$$

where PG_t denotes the deflated Gulf oyster price in quarter t expressed in dollars per pound of meats (1982–1984 Consumer Price Index equals base); QG_t denotes the Gulf oyster harvest in quarter t expressed in millions of pounds of meats; INC_t denotes the U.S. real disposable income in quarter t expressed in billions of dollars; VUL_t is a binary variable used to capture the expected change in demand associated with warning labels and associated media attention (equal to 0 before 1991 and 1 thereafter); $SEAS_t$ is a binary variable used to capture seasonality in the demand for Gulf oysters (equal to 0 for the months April through September, i.e., the 2nd and 3rd quarters, and 1 for all other months, i.e., the 1st and 4th quarters); $LATX_t$ denotes the combined Louisiana and Texas share of Gulf harvest in quarter t; LPG_t denotes the deflated Gulf price lagged one quarter; and ϵ_t denotes the error term. Parameters to be estimated range from β_0 to β_8.

The price-dependent specification of equation 1 reflects the fact that production in the Gulf tends to be primarily determined by the availability of oysters, which, in turn, is largely dictated by environmental influences. This would suggest that price would be more responsive to changes in quantity than vice versa. From a theoretical perspective, one would anticipate a negative price response to a change in Gulf harvest (QG_t), i.e., $\partial PG_t / \partial QG_t < 0$. As specified, furthermore, the response in price to a change in the quantity harvested is permitted to vary by season. In the 2nd and 3rd quarters, defined for purposes of this study as the summer months, the impact of a one-million-pound change in QG_t is equal to β_1. In the 1st and 4th quarters, the impact is equal to $\beta_1 + \beta_6$.

Assuming oysters to be a normal good, price is anticipated to respond positively to increases in real disposable income (INC_t). The variable VUL_t, as noted, was included to capture any decrease in demand associated with increased information (i.e., warning labels and media), and as such, the parameter β_3 is hypothesized to be negative. Because the incidence of *V. vulnificus* is temperature dependent and is higher in the warmer months of the year, it is further hypothesized that the impact of VUL_t may vary by season with the impact on demand being greater in the summer months. Therefore, an interaction term between $SEAS_t$ and VUL_t is included in equation 1. In the colder months, i.e., the 1st and 4th quarters of the year, which for purposes of this study are defined as the winter months, the impact of VUL_t on dockside price (PG_t) is equal to $\beta_3 + \beta_5$. In the summer months, i.e., the 2nd and 3rd quarters, the impact of VUL_t on dockside price is equal to β_3. Given that the impacts of VUL_t on PG_t are anticipated to be higher when temperatures are warmer, one would anticipate that β_5 is positive, implying that the absolute value of β_3 exceeds the absolute value of $\beta_3 + \beta_5$.

The variable $SEAS_t$, as previously noted, was included in the analysis to capture the hypothesized seasonality component in Gulf oyster demand. Although the adage "don't eat oysters in any month without an 'R' " may have little validity, particularly since refrigeration of the oyster product became the norm, some adages tend to endure the test of time, and, though not documented, it is thought that demand for oysters is lower in the warmer months of the year, *ceteris paribus*. Furthermore, as noted, demand in the summer months since the introduction of warning labels is anticipated to be below that of the preceding period. Differentiating equation 1 with respect to $SEAS_t$ (i.e., $\partial PG_t / \partial SEAS_t$) yields $\beta_4 + \beta_5 VUL_t + \beta_6 QG_t$. This would suggest that the term $\beta_4 + \beta_6 QG_t$, i.e., the impact of seasonality before increased information, is less than zero, and furthermore, that the impact of seasonality after the initiation of warning labels ($\beta_4 + \beta_5 VUL_t + \beta_6 QG_t$) exceeds that before the increased information.

Based on examination of reported dockside oyster prices by state (National Marine Fisheries Service 2000), there appears to be a premium attached to the price of oysters harvested in Louisiana and Texas (these two states represent the majority of Gulf production), perhaps due to a larger average size. Hence, one would expect that the average Gulf price is positively related to the share of production derived from these two states. The variable $LATX_t$ is included in equation 1 to capture the price effect associated with the apparent product heterogeneity.

The variable LPG_t is included in the Gulf dockside price model (equation 1) in an effort to capture any inertia in the change in dockside price to changes in exogenous variables. The value of β_8 is expected to fall between 0 and 1, with a value approaching 0 indicating instantaneous adjustment in price to changes in the value of exogenous variables whereas a value approaching 1 suggests a high degree of inertia.

Finally, substitute products are often entered as exogenous variables in demand models of this nature. One would hypothesize that oysters produced in other regions of the country would constitute the closest substitutes for Gulf oysters. Initial inclusion of Chesapeake harvest in the Gulf demand equation did not prove successful; hence, it was not included in the final version of the model. This finding is consistent with other information presented by Diop and Keithly (2000).

Chesapeake Demand Model

Corresponding to the demand equation for the Gulf oysters (equation 1) is a demand equation for the Chesapeake oysters specified as

$$PC_t = \alpha_0 + \alpha_1 QG_t + \alpha_2 QC_t + \alpha_3 INC_t + \alpha_4 VUL_t + \alpha_5 SEAS_t + \alpha_6 (SEAS^* VUL)_t + \alpha_7 (QC^* SEAS)_t + \alpha_8 LPC_t + \alpha_9 (QG^* VUL)_t + \alpha_{10} PFI_t + \xi_t \quad (2)$$

where PC_t denotes the deflated Chesapeake dockside oyster price in quarter t expressed in dollars per pound of meats (1982-1984 Consumer Price Index equals base); QC_t denotes the harvest of Chesapeake oysters in quarter t expressed in millions of pounds of meats; LPC_t denotes the deflated Chesapeake price lagged one quarter; PFI_t is a binary variable used to capture the incidence of *Pfiesteria* in the Chesapeake, which may have influenced the demand for Chesapeake product (equal to zero before the third quarter of 1997 and one thereafter); ξ_t represents the error term in quarter t; and α_0 through α_{10} are the parameters to be estimated. For reasons analogous to those given for the Gulf, the Chesapeake demand equation is specified as price dependent.

As with the Gulf demand model, one would expect the Chesapeake dockside price to be negatively related to own landings (i.e., $\partial PC_t/\partial QC_t < 0$) and the landings of the Gulf substitute product (i.e., $\partial PC_t/\partial QG_t < 0$). As specified, the impact of Gulf production on the Chesapeake dockside price is permitted to vary after the increased information attached to the Gulf product. As discussed in greater detail below, certain restrictions were imposed on the Chesapeake demand model (equation 2) so that the Gulf production had no impact on the Chesapeake price after 1990.

The impact of Gulf product warning labels and the associated media attention on the demand for the Chesapeake product is, *a priori*, uncertain. If consumers have perfect knowledge and act rationally, then one would hypothesize that warning labels and media associated with the Gulf raw product should lead to an increase in demand for the Chesapeake product, *ceteris paribus*. Conversely, if consumers have less than perfect information or do not act rationally, one could hypothesize that the Chesapeake price has been negatively affected by actions taken with respect to the Gulf product. Differentiating equation 2 with respect to VUL_t (i.e., $\partial PC_t/\partial VUL_t$) yields $\alpha_4 + \alpha_6 SEAS_t + \alpha_9 QG_t$. If negative, one would conclude that the Chesapeake price has been negatively affected from the increased information attached to the Gulf product. Although the direction and degree of impact is *a priori* uncertain, Strand's (1999) synthesis suggests that it is likely to be negative.

DATA AND ESTIMATION ISSUES

Data Issues

The Gulf dockside demand model (equation 1) and corresponding Chesapeake demand model (equation 2), developed and specified in the previous section, were estimated based on quarterly data covering the 1981–1997 period. All landings and price data were derived from unpublished data provided by the National Marine Fisheries Service. Some summary statistics, relevant to quantity and price variables included in the respective models, are presented in Table 1.

The deflated Gulf oyster price averaged $1.64 per pound during the 1981–1997 period but only $1.33 per pound since 1991. This is a reduction of almost 30% from the average reported price of $1.85 during 1981–1990. The quantity harvested averaged 5.2 million pounds per quarter during the period of analysis, with the pre-1991 quarterly production (5.4 million pounds) averaging approximately 10% more than the post-1990 production (4.9 million pounds). In general, little price variation was evident during the 1981–1997 period when examined on a seasonal basis even though production during the winter season, which averaged 6.1 million pounds per quarter, exceeded the production during the summer season, which averaged 4.3 million pounds per quarter, by approximately 40%. For the 1991–1997 period, winter season production averaged 5.7 million pounds per quarter compared with 4.2 million pounds per quarter in the summer season.

The deflated Chesapeake price averaged $1.95 per pound during 1981–1997 period based on average quarterly production of 1.8 million pounds (Table 1). Before 1991, the deflated Chesapeake price averaged $1.88 per pound, or approximately 2% above that reported in the Gulf, based on average quarterly production of 2.8 million pounds. After 1990, the quarterly deflated Chesapeake

TABLE 1.

Summary statistics pertaining to Gulf of Mexico and Chesapeake Bay oyster demand models.

	Overall Mean	Winter Mean	Summer Mean
1981–1997			
PG ($/lb)	$1.64 (0.51)[a]	$1.61 (0.52)	$1.66 (0.50)
PC ($/lb)	$ 1.95 (0.41)	$ 2.07 (0.38)	$ 1.82 (0.40)
QG (Mill lbs)	5.20 (1.91)	6.11 (2.08)	4.28 (1.17)
QC (Mill lbs)	1.82 (2.38)	3.06 (2.82)	0.58 (0.65)
1981–1990			
PG ($/lb)	$ 1.85 (0.51)	$ 1.82 (0.54)	$ 1.88 (0.49)
PC ($/lb)	$ 1.88 (0.49)	$ 2.08 (0.49)	$ 1.69 (0.43)
QG (Mill lbs)	5.38 (2.20)	6.40 (2.48)	4.36 (1.27)
QC (Mill lbs)	2.82 (2.67)	4.71 (2.61)	0.94 (0.63)
1991–1997			
PG ($/lb)	$ 1.33 (0.29)	$ 1.31 (0.28)	$ 1.34 (0.31)
PC ($/lb)	$ 2.03 (0.22)	$ 2.07 (0.15)	$ 2.00 (0.27)
QG (Mill lbs)	4.94 (1.40)	5.71 (1.31)	4.20 (1.05)
QC (Mill lbs)	0.39 (0.44)	0.70 (0.43)	0.07 (0.04)

[a] Standards errors of means are given in parentheses. PG and PC are respectively deflated Gulf of Mexico and Chesapeake oyster prices in quarter t. QG and QC are respectively the harvest of Gulf of Mexico and Chesapeake oysters in quarter t.

price averaged $2.03 per pound, based on quarterly production of approximately 390,000 pounds. Overall, the deflated Chesapeake price during 1991–1997 exceeded the Gulf deflated price for the same period by approximately 20%.

Estimation Issues

Three estimation issues pertinent to the current analysis—the treatment of lagged dependent variables in the analysis, the use of prior restrictions in the estimation process, and contemporaneous correlation of the error terms—are briefly reviewed below.

The Use of Lagged Values

The lagged Gulf and Chesapeake dockside prices (LPG_t and LPC_t, respectively), as noted, were included in the analysis based on the premise that the response in price to a change in an exogenous variable may not be completed during that quarter in which the change in the exogenous variable occurred, i.e., there exists some inertia in the change in price. Pindyck and Rubinfeld (1991) and Green (2000), who provided a complete discussion of the distributed lag models, derived the following infinite lag distribution that is relevant to the oyster model presented in the previous section:

$$Y_t = \alpha(1 - w) + wY_{t-1} + \beta X_t + (\varepsilon_t - w\varepsilon_{t-1}) \qquad (3)$$

where w is the lag weight assumed to be positive and declining geometrically. The implications associated with equation 3 are twofold. First, all past values of the exogenous variable (X) are captured in the endogenous variable (Y) lagged one period with the impact of a change in X on Y decaying at a geometric rate over time. Second, lagging the dependent variable results in the introduction of serial correlation of the error term, assuming ε_t in equation 3 does not exhibit an autocorrelation pattern.

Several methods have been proposed for estimating the geometric lag structured model in the presence of serial correlation. The most popular technique, and the one that is used in the current analysis, is the instrumental variable approach whereby an estimate of the lagged dependent variable is generated by regressing its value against the lagged values of the exogenous variables in the model. Then the model is estimated using a maximum likelihood procedure, which corrects for serial correlation.

Given the structure of a geometric lag model, it is useful to identify both the mean lag as well as the long-run impact associated with a permanent change in the level of an exogenous variable. The mean lag, defined as the amount of time that expires before one-half of the impact on the dependent variable is recognized in association with a one-time change in an exogenous variable, is equal to $w/(1 - w)$. In our case, w is the estimate associated with the Gulf or Chesapeake dockside prices lagged one period (i.e., β_8 and α_8, respectively). The long-run response to a change in an exogenous variable is equal to $\beta/(1 - w)$. Hence, as the value for w increases ($0 < w < 1$), the greater will be the amount of time, which expires before the full impact of a one-time change in an exogenous variable is recognized.

As a concrete example, let us examine the impact of a change in the dockside demand for Gulf and Chesapeake oysters in relation to a change in real disposable income (INC_t). Specifically, the immediate impact of a one-billion-dollar change in income on the dockside demand for Gulf oysters is equal to the parameter estimate β_2 (see equation 1), whereas an equivalent income change immediately affects the dockside demand for Chesapeake oysters by α_3 (see equation 2). However, the full impact of a change in income in the current time period (i.e., quarter t) may not be realized in the current period due to inertia in consumers varying their spending patterns (i.e., it may take several quarters for consumers to fully adjust their spending patterns as a result of a current change in income). With respect to the Gulf, the long-run change in demand in response to a one-billion-dollar change in income in the current period is equal to $\beta_2/(1 - \beta_8)$. For the Chesapeake, the long-run response is equal to $\alpha_3/(1 - \alpha_8)$. It can be easily demonstrated that as β_8 and α_8 approach zero, the long-run response to a change in income will approach the immediate response.

Prior Restrictions

Cointegration and vector autoregression analysis conducted by Diop and Keithly (2000) provided strong evidence that the Gulf market strongly influenced the Chesapeake market before 1991 (measured via the Chesapeake dockside price). After 1990, however, the Gulf market had little or no influence on the Chesapeake market, i.e., the markets became separate. Based on these findings, the parameters α_1 and α_6 were restricted to equal one another but be of opposite sign. Imposition of this restriction results in changes in Gulf production after 1990 having no impact on the Chesapeake dockside price. This is justified given the apparent separation of the two markets after institution of warning labels and the negative publicity associated with the Gulf product.

Seemingly Unrelated Regression (SUR)

The dockside oyster demand equations for the Gulf and the Chesapeake bear a close conceptual relationship with each other. Hence, one would expect there to be a correlation in the error terms between the two equations. As such, SUR was used to increase efficiency of the estimates. The analysis was conducted using PROC MODEL of the SAS/ETS Software (SAS Institute Inc. 1993).

EMPIRICAL RESULTS AND DISCUSSION

A summary of the regression results associated with the estimated Gulf and Chesapeake dockside demand models is presented in Table 2.

Gulf Demand Model

The estimated parameters in general agreed with prior expectations, and, with few exceptions, all estimated parameters were significant at the 90% confidence level. The estimated model explained approximately 91% of the variation in the dependent variable (Table 2).

To examine the impact of a change in harvest on the deflated dockside price, differentiate equation 1 with respect to QG_t, which, for the parameter estimates given in Table 2, yields

$$\partial PG_t/\partial QG_t = -0.1198 + 0.0591SEAS_t \qquad (4)$$

This suggests that a one-million-pound increase (decrease) in summer harvest results in an immediate $0.1198 decrease (increase) in the deflated Gulf dockside oyster price, which, when converted to 1997 dollars, is equivalent to a $0.197 decrease (increase). An equivalent change in the winter harvest, by comparison, results in

TABLE 2.

Estimated parameters and standard errors associated with Gulf and Chesapeake oyster demand models. Asterisk indicates parameter estimates significant at the $\alpha = 0.10$ level.

Variables	Gulf Demand		Chesapeake Demand	
	Estimated Parameters	Asymptotic Standard Error	Estimated Parameters	Asymptotic Standard Error
Intercept	0.4485	0.3131	1.7206*	0.7536
QG_t	−0.1198*	0.0229	−0.1024*	0.0223
QC_t	—	—	−0.2075*	0.1124
INC_t	0.332E-3*	0.137E-3	−0.150E-4	0.218E-4
VUL_t	−0.5367*	0.1097	−0.3600*	0.1346
$SEAS_t$	−0.3852*	0.1215	0.5876*	0.1294
$(SEAS*VUL)_t$	0.1406*	0.0736	−0.5673*	0.1431
$(QG*SEAS)_t$	0.0591*	0.0222	—	—
$(QC*SEAS)_t$	—	—	0.1626	0.1018
$LATX_t$	0.0999	0.2504	—	—
LPG_t	0.5603*	0.0814	—	—
LPC_t	—	—	0.3577*	0.1188
$(QG*VUL)_t$	—	—	0.1024*	0.0223
PFI_t	—	—	−0.0321	0.1691
Adjusted R^2	0.906	—	0.669	—
Durbin Watson (DW)	2.024	—	1.736	—

an immediate inverse price response of only $0.0607 per pound ($0.097 in 1997 dollars), or approximately one-half of that estimated for the summer season. The short-run price flexibility of demand with respect to harvest, which measures the change in price to a 1% change in harvest, was found to equal −0.309 in the summer season compared with −0.230 in the winter season. Hence, a 10% increase in the summer harvest is expected to result in an immediate reduction in the dockside price of 3.09%, whereas an equivalent increase in the winter harvest results in a 2.30% reduction in price. These figures suggest that the immediate price response to a change in harvest is relatively inflexible.

The structure of the model makes it important to differentiate an immediate impact from the long-run impact. In the long-run, a one-million-pound increase (decrease) in summer harvest was found to result in a $0.272 decrease (increase) in the deflated Gulf of Mexico dockside price ($0.436 in 1997 dollars), whereas a one-million-pound increase (decrease) in the winter harvest was estimated to result in a price decrease (increase) of $0.138 per pound ($0.221 in 1997 dollars). The mean lag length is equal to approximately 1.25 periods [i.e., 0.560/(1 − 0.560)], implying that one-half of a permanent change in landings in quarter t will be felt within the initial 1.25 quarters. The long-run price flexibility with respect to summer harvest was estimated to equal −0.702 compared with −0.523 with respect to winter harvest. Hence, the price response to harvest is inflexible even in the long run, which implies that increasing harvest will result in an increase in revenues to the fishermen.

The increased risk information provided since 1991 was found to significantly impact the Gulf oyster dockside demand in a negative manner, with the degree of the impact depending on season. To examine the impact, differentiate equation 1 with respect to VUL_t, which, for the parameter estimates given in Table 2, yields

$$\partial PG_t/\partial VUL_t = -0.5367 + 0.1406SEAS_t \qquad (5)$$

Warning labels and associated media attention resulted in an estimated reduction in the summer Gulf dockside price of $0.5367 per pound ($0.859 per pound in 1997 dollars) since 1991 compared with a reduction in the deflated winter price of $0.3961 per pound ($0.634 per pound in 1997 dollars). These reductions, however, reflect only the initial effect of the warning labels and media attention by season. The fact that the estimate of β_8, equal to 0.560, falls between 0 and 1 implies that as one moves further away from the date that warning labels were initially mandated, the greater the absolute value of the magnitude of the policy variable. In the long run, the impact of warning labels was estimated to result in a summertime reduction in the demand for Gulf oysters equivalent to $1.22 per pound ($1.95 per pound in 1997 dollars) and a wintertime reduction in demand equal to $0.901 per pound ($1.44 in 1997 dollars). The actual summer price in 1997 equaled $2.16 whereas the actual winter price equaled $2.22, suggesting that the summer price was reduced by nearly one-half as a result of the warning labels and media attention whereas the winter price was reduced by approximately 40%.

One could hypothesize that the impact of warning labels and the associated media attention decays at some rate with the passage of time as consumers either forget about the negative publicity or overcome initial fears. To examine whether this was the case, the analysis was also conducted for the 1981–1995 period. In general, the parameter estimates varied only marginally, suggesting that the decay in the initial impact is, at most, minor. This finding may reflect the fact that warning labels are permanent in nature; hence, the information attached to these warning labels continuously reinforces consumer perception that the consumption of raw oysters is "risky."

Differentiating equation 1 with respect to $SEAS_t$ yields the following equation based on the parameter estimates given in Table 2:

$$\partial PG_t/\partial SEAS_t = -0.3852 + 0.1406VUL_t + 0.0591QG_t \qquad (6)$$

The result of substituting the average pre-1991 winter harvest (i.e., 6.4 million pounds) into equation 6 suggests almost no consumer demand differential between winter and summer Gulf oyster

demand before the institution of warning labels and media attention, *ceteris paribus*. As indicated in Table 1, the observed summer price exceeded the winter price by $0.05 per pound before 1991. Given the results of the current analysis, it appears that the higher observed summer price reflects a lower level of production in the summer season. After 1991, in association with the mandatory warning labels and media attention, the difference in demand between the winter season and the summer season resulted in an expected short-run price differential of approximately $0.075 per pound ($0.12 per pound in 1997 dollars). In the long run, the price differential increases to $0.151 ($0.24 per pound in 1997 dollars).

Income, as indicated in Table 2, was found to significantly influence the Gulf oyster dockside demand with a $100 billion-dollar increase in real disposable income resulting in an immediate $0.033 increase in price and a $0.075 increase in price in the long run. The estimated long-run price flexibility with respect to income equaled 1.42, suggesting that a 1% increase in real disposable income results in a price increase in excess of 1% in the long run, *ceteris paribus*.

Chesapeake Demand Model

As with the Gulf demand model, the Chesapeake demand model appears to perform adequately based on the expected signs and statistical significance associated with most variables included in the equation. The adjusted R^2 associated with the Chesapeake model equaled 0.67 (Table 2).

For the parameter estimates in Table 2, differentiating equation 2 with respect to a change in QC_t yields

$$\partial PC_t/\partial QC_t = -0.2075 + 0.1626SEAS_t \quad (7)$$

It can therefore be concluded that a one-million-pound increase (decrease) in the Chesapeake summer harvest results in an immediate $0.2075 decrease (increase) in the deflated Chesapeake price ($0.332 in 1997 dollars) whereas a similar increase (decrease) in the Chesapeake winter harvest results in an immediate $0.0449 decrease (increase) in the deflated price ($0.072 in 1997 dollars). The long-run impact of a one-million-pound increase (decrease) in the Chesapeake summer harvest was found to result in a $0.323 decrease (increase) in Chesapeake price, which is considerably greater than the long-run impact of a similar change in the winter harvest ($0.0699, or $0.112 in 1997 dollars). The short-run price flexibility of the summer Chesapeake price with respect to own landings was found to equal 0.066 (0.10 long-run price flexibility), which is identical to that estimated with respect to the winter harvest.

Differentiating equation 2 with respect to a change in Gulf quantity yields

$$\partial PC_t/\partial QG_t = -0.1024 + 0.1024VUL_t \quad (8)$$

Before 1991, therefore, one would expect the deflated Chesapeake price to immediately decrease (increase) by $0.1024 ($0.164 in 1997 dollars) in response to a one-million-pound increase (decrease) in Gulf production. The long-run response would be $0.159. Given the restriction imposed on the Chesapeake demand model (based on separate markets after 1990), changes in Gulf landings after 1990 had no impact on the Chesapeake dockside price.

Changes in the deflated Chesapeake dockside price in response to the mandated warning labels on the Gulf product (and associated negative publicity) can be evaluated by differentiating equation 2 with respect to VUL_t, which yields

$$\partial PC_t/\partial VUL_t = -0.3600 - 0.5673SEAS_t + 0.1024QG_t \quad (9)$$

Based on the mean level of summer Gulf landings during 1991–1997 (4.2 million pounds), the impact of warning labels and media attention attached to the Gulf product was found to result in an immediate price increase in the deflated Chesapeake dockside summer price equal to $0.0496 per pound ($0.079 in 1997 dollars). The Chesapeake winter price, however, was estimated to decline by $0.374 per pound ($0.60 in 1997 dollars) as a result of warning labels and media attention attached to the Gulf product. Overall, one can conclude that the increased summer price reflects the fact that, given the structure of the model, the Gulf product does not compete with the Chesapeake product after 1990; hence, the lack of the substitute product for the Chesapeake product in the summer months results in an overall increase in the Chesapeake price. The long-run impact of warning labels and media attention on the Chesapeake dockside equaled $0.077 in the summer months ($0.124 in 1997 dollars) and resulted in a reduction in the "winter" price equal to $0.564 per pound.

Season ($SEAS_t$) was found to strongly influence demand for the Chesapeake product. Based on the mean level of Chesapeake winter production before 1991 (4.71 million pounds), the results suggest that higher demand in the winter months resulted in a price increase of $1.35 per pound ($2.16 in 1997 dollars) when compared with the summer price (before 1991). The price differential narrowed to only $0.16 per pound after the 1991 events in the Gulf.

The parameter estimate associated with disposable income in the Chesapeake equation was found to be negative but statistically insignificant. The unexpected sign associated with INC_t may reflect the strong influence that the Gulf product has on the Chesapeake price.

The parameter estimate associated with pfisteria (PFI_t) was found to be negative but statistically insignificant in determining the demand for the Chesapeake oyster (Table 2). Because PFI_t was observed in the Chesapeake for a very short period of time, this may explain why it was not a factor in explaining the changes in Chesapeake oyster demand.

Finally, the mean lag length in the Chesapeake market was found to equal 0.55 periods [i.e., 0.3577/(1 − 0.3577)], implying that one-half of a permanent change in Chesapeake landings in quarter t will be felt within 0.55 periods of the first quarter. This implies that the lagged Chesapeake price plays the role of a propagation mechanism that carries over the remainder of the change from one quarter to the next.

Revenue Implications

The results presented here can be used to assess the welfare implications associated with increased consumer awareness associated with the consumption of raw oysters. For example, since 1991, the demand for Gulf oysters has shifted downward, leading to lower harvested quantities and prices. The estimated dockside equations were used to quantify the welfare effects of *V. vulnificus* on the oyster industry. Quarterly predictions of prices with increased consumer awareness were compared with simulated prices in the absence of warning labels and media attention. For the period covering 1991 through 1997, the difference in prices multiplied by the corresponding quantities represented the overall loss in revenue to oyster harvesters. The overall industry loss in revenue (in 1997 dollars) due to increased consumer information amounted to $16.4 million, with the Gulf producers incurring

about 95% of the losses and the Chesapeake producers the remaining 5%. The $7.82 million loss for the Gulf producers during the summer months represented 50% of the overall $15.6 million loss for that region. The Gulf harvesting sector bore most of the losses because it is the primary oyster-producing region in the United States.

ACKNOWLEDGMENTS

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NATIONAL INDICATOR STUDY: IS AN INTERNATIONAL APPROACH FEASIBLE?

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ABSTRACT The National Indicator Study (NIS) was established in the United States in 1989 with the purpose of improving the shellfish classification systems for molluscan shellfish growing waters using the latest technological advances in microbiological and epidemiological methods. A total of ten methods development projects were completed, eight addressing indicators of pathogens associated with molluscan shellfish and two with direct detection of specific viral pathogens, Norwalk and hepatitis A. In 1993, the results of all ten projects were submitted to peer review by a panel of scientists. Two of these candidate indicators were selected for further development: particulate-bound secretory immunoglobulin (sIgA) and the male-specific coliphage. This paper reviews the funded projects, the Literature Review and Manual of Methods that were funded by the study, and raises the question: should an international indicator study be pursued, by whom, and through what sources of funding? Unfortunately, the NIS was discontinued in 1994 before it achieved any quantitative information on the efficacy of these new or existing indicator methods as they might relate to the improvement of water classification procedures or health risks to consumers.

KEY WORDS: National Indicator Study, molluscan shellfish, National Shellfish Sanitation Program, Interstate Shellfish Sanitation Conference

INTRODUCTION

The current system used to determine the suitability of molluscan shellfish (oyster, clam, and mussel) harvest waters to produce a raw product safe for human consumption originated in 1925. The management system was developed by the U.S. Public Health Service working cooperatively with state health departments to control typhoid and cholera outbreaks that were associated with the consumption of raw shellfish. Because the actual control of molluscan shellfish in interstate transport is the responsibility of the states, they, in turn, formed the Interstate Shellfish Sanitation Conference (ISSC). The ISSC, in cooperation with the U.S. Food and Drug Administration, developed the National Shellfish Sanitation Program (NSSP), which provides criteria for the protection of harvest waters, harvesting, transport, and processing of shellfish (ISSC et al. 1999).

At the harvest level, the NSSP provides guidance for states conducting sanitary surveys in the harvest areas. These sanitary surveys are used to assess water quality and determine the suitability of shellfish for human consumption. The shoreline survey and bacteriological examination of molluscan shellfish waters for indicator organisms are routinely performed in harvest areas. However, when harvest areas are subject to intermittent pollution events, states must exercise more intensive monitoring after the event to ensure that the shellfish are cleansed of indicator organisms. Some shellfish waters are used as sources for natural or mechanically controlled cleansing of shellfish (relay or depuration operations, respectively).

The shoreline survey is the most important step in the approval of waters for direct harvest. The survey involves (1) physical examination of properties and facilities that may contribute pollution of public health significance; (2) review of relevant discharge permits; (3) use of dye studies, dispersion models, and other hydrographic techniques to evaluate the dispersion and dilution of point source effluent; (4) evaluation of treatment reliability at major waste water treatment plants; (5) evaluation of agricultural best management practices (BMPs) for animal waste containment and similar activities directed at pollution identification and control. The successful completion of shoreline surveys often requires the skills, resources, and cooperation of several state and local agencies. These surveys provide the framework for assessing the significance of indicator organisms found in bacteriological examination of shellfish waters and meats.

The bacteriological examination of shellfish harvest waters is used to verify information obtained from shoreline surveys. Elevated levels of indicator organisms in approved harvest areas indicate a need for shoreline survey reexamination. Between shoreline surveys, monitoring of indicator organisms can confirm that no significant new sources of human sewage were introduced into a harvest area. The NSSP states that the determination of the acceptability of a harvest area should be based on both the shoreline survey and water quality sampling information.

During recent years the suitability and credibility of the existing coliform indicator have become increasingly questionable, particularly with respect to the transmission of viral illness. Consumers, scientists, state and federal regulators, and industry representatives are questioning whether the current indicators and the existing numeric criteria adequately predict the risk of illness transmission to consumers (Hackney 1993b).

DO WE NEED AN INTERNATIONAL INDICATOR STUDY?

In all countries where raw molluscan shellfish products are commercially available, the public expects those products to be safe for human consumption. Public health agencies and resource agencies within shellfish-producing countries are charged with determining the acceptability of shellfish harvest waters as safe sources of food. These agencies need better analytical tools and microbial standards to improve their performance in the evaluation of the safety of shellfish harvest waters. Currently, there are different indicators and protocols used throughout the shellfish-producing world, and these are sometimes perceived as trade barriers. Of concern to the U.S. industry is the lack of a level playing field to facilitate shellfish trade (Leonard 1992b). Also, the molluscan shellfish industry needs assurance that it is marketing a safe product from safe waters. Better indicators of the safety of molluscan shellfish for consumption will help provide that assurance.

Uncertainties related to the current indicators have also led to concerns that their use may result in harvesting restrictions in areas where human health risk is minimal. As a result, recreational and commercial harvesters, as well as some agency regulators, believe

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the public may be unjustly deprived of resource use. This belief is particularly strong with respect to areas affected primarily by runoff from fields, forests, and marshes where there is little or no human habitation. In other situations, it is believed that impacts from human sources that may cause a human health risk remain undetected because of the inadequacies of the current coliform indicator (Hackney 1993b).

The question arises as to why the search continues for new indicators. Why not concentrate on testing for the pathogens of concern? The reasons for not pursuing specific pathogen isolation are many. First, specific pathogen isolation is not cost effective. Methodologies are cumbersome, time consuming, and expensive. Several of the important illnesses associated with shellfish consumption are diagnosed by the symptoms the pathogen induces in people because no method exists for pathogen isolation. Secondly, pathogens occur in fewer numbers in fecal material than other microbial organisms, particularly in the combined sewage wastes from large populations. When the wastes are discharged into or near harvest waters and then diluted, the probability of capturing the pathogenic organisms in a small sample size diminishes rapidly.

Another complication in direct pathogen isolation is that pathogens are not evenly distributed in shellfish harvest waters. Failure to detect a pathogen in a specified volume of water (a sample) is not proof that the pathogen is absent in the larger water body from which the sample was taken. It only verifies that the pathogen was not detected in that set of samples. Furthermore, the presence or absence of a particular pathogen does not necessarily correlate with the presence or absence of another pathogen. The lack of this correlation among pathogens would require testing for all pathogens, a task that is not economically feasible.

In the United States, the coliform indicator has been fairly effective in predicting the presence of fecal-borne bacterial pathogens. However, in the last 30 years there have been increasing disease outbreaks from viral rather than bacteriological pathogens, and the coliform indicator may not be at all effective to determine absence/presence and concentration of viruses. In recent years, the ISSC has focused their efforts on reducing confirmed disease cases resulting from naturally occurring pathogens in estuarine waters such as *Vibrio vulnificus* and *Vibrio parahaemolyticus* and the impacts of harmful algal blooms. In these cases, the fecal coliform indicator cannot be used to determine pathogen or toxin levels.

THE NIS CHRONOLOGY

In the summer of 1987, a group of twenty scientists and managers met in Cocodrie, Louisiana to design a study that would result in improved risk management of the sale and consumption of raw molluscan shellfish. The incentive for this memorable meeting came from the Gulf oyster industry, which had lost confidence in the NSSP and felt an obligation to its consumers to better ensure the safety of its products. Conditions were a little rough at the unfinished facility of the Louisiana Universities Marine Consortium (LUMCON), but the study design group was so successful in developing a plan and cost estimates that they still form the core of the NIS. The purpose of the NIS was to improve existing classification systems for molluscan shellfish–growing waters using the latest technological advances in microbiological and epidemiological methods. The goal was a long-term research program to develop and evaluate the effectiveness of new indicators of health risk associated with consumption of raw molluscan shellfish.

The NIS was managed in cooperation with the ISSC and the LUMCON. The ISSC provided oversight and developed the goals and objectives of the study. The NIS Committee (NISC) of the ISSC was established in May 1991 under a Memorandum of Understanding between the National Marine Fisheries Service (NMFS) and the ISSC. The ISSC and LUMCON reached agreement in December 1991 whereby LUMCON would provide scientific management on behalf of the NISC. A contract between NOAA and LUMCON was executed in July 1992 to provide an appropriate funding mechanism to support LUMCON's activities, including subcontracted research.

With this administrative structure now in place, the NISC continued to follow a course of work in accordance with the original plans of the NIS, i.e., to develop and test new and improved methods for measuring human-specific indicators of fecal pollution in molluscan shellfish–growing waters. Methods development was considered the first phase of a much larger research program that would eventually be directed to quantitative assessment of the health risk to shellfish consumers. Consequently, LUMCON was requested to develop, via subcontract, improved procedures for measuring sewage-related microorganisms in shellfish meats. The research was halted in June 1994, following the announcement of the discontinuance of the NIS. The NISC met for the final time in February 1994, at which time its members were advised that no further funding would be made available to support the study and the NIS would be closed down within the current fiscal year.

RESEARCH RESULTS

A total of ten methods development projects were completed over the entire course of the NIS since its inception in 1989. Of these, eight dealt specifically with indirect indicators of pathogens associated with molluscan shellfish and two with direct detection of specific viral pathogens, Norwalk and hepatitis A.

The results of all 10 projects were subjected to peer review by an independent panel of scientists convened by the NISC in September 1993. The panel was charged with reviewing the research for technical merit and completeness of data, its applicability and utility, and its consistency with NIS objectives. The panel was also asked to offer recommendations on the NIS strategy, including additional research to identify, isolate, culture, and quantify indicator organisms.

The review panel concluded that, among the various indicator methods studied, two should be considered as candidates for further development as human-specific indicators of fecal contamination. The most promising candidate was found to be particulate-bound secretory immunoglobulin (sIgA), a chemical indicator that could be used to identify nonpoint sources of pollution, such as septic systems (human sIgA) and wildlife wastes (animal sIgA). The other strong candidate was male-specific coliphage, which can be serotyped to distinguish between human and animal sources of pollution. Specific recommendations of the review panel concerning these two methods and the other research approaches were summarized in a draft report prepared by the NISC in January 1994 (ISSC 1994).

NIS-Funded Research Projects

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Chang, G. Practical Use of High Tech Concepts: Human-Specific *E.coli* and Rapid Detection of *E.coli* and Fecal Coliform.

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NIS PRODUCTS

Hackney, C. R. and M. D. Pierson, editors (1994) *Environmental Indicators and Shellfish Safety*

An early objective of the NIS was to prepare a comprehensive literature review of environmental indicators and shellfish safety so as to provide a framework for evaluating the efficacy of current indicators and seeking ways to improve or replace them for use in the classification of molluscan shellfish growing waters. The review, titled *Environmental Indicators and Shellfish Safety*, was published in 1994 in book form. It is also available in electronic form upon request through the ISSC. The book begins with the history of shellfish safety. This chapter was written by the former head of the U.S. FDA's Shellfish Sanitation Branch and contains much information, before only available as FDA files. The second chapter is on microbial and chemical indicators of pollution. This is perhaps the most comprehensive chapter ever written on the subject. The author of this chapter, Howard Kator, has been the chairman of the ISSC microbiology committee for several years and has had considerable research experience on the relationship between environmental indicators and pathogens. The next four chapters examine shellfish safety with respect to enteric viruses and bacterial pathogens, and their relationship to environmental indicators. These chapters present characteristics of the organisms, their survival in the environment, the methodology to recover the organisms, and their relationship to environmental indicators and human illness. The next three chapters concentrate on the methodology, including rapid methods and the emerging methodology. The phenomenon of cell injury is reviewed along with viable but nonculturable cells. Following this section, the changes in indicator and pathogen populations during handling or storage of shellstock and during processing are reviewed. The last four chapters of the book review sanitary surveys of growing waters, depuration and relaying of molluscan shellfish using indicator information for managing risks, epidemiological studies, and statistical sampling of growing waters and shellfish meats.

Hackney, C. (1994) *Reported Human Illness from Consumption of Molluscan Shellfish in the United States from 1973–1990*

This report summarized data from the U.S. Centers for Disease Control (CDC) and the U.S. Food and Drug Administration's North East Technical Services Unit (NETSU) concerning reported human illness associated with the consumption of molluscan shellfish. The CDC database included the years 1973 to 1987, whereas the NETSU database included 1973 to 1990. The databases do not agree because of the different sources of information used to compile each. The CDC database is the annual summaries of outbreaks and cases reported by the states, one of three data sources for information from CDC. Other information sources include the Laboratory Surveillance System and the CDC publication *Mortality and Morbidity Weekly*. The Laboratory Surveillance System mostly targets specific organisms such as *Salmonella* spp. The CDC and NETSU databases also differ in the definition of shellfish. In the CDC database, shellfish is broadly defined, and includes crustaceans, bivalves, and univalves. In the NETSU database, shellfish is much more narrowly defined as bivalve mollusks. Also, the NETSU database is very specific as to shellfish species, illness, etc. Each case or outbreak is briefly described. The information given includes location of illness, location of harvesting state if known, the shellfish species, the bacterial or viral agent, the reference cited, and brief comments on the case.

The NETSU database is a summary of cases, not outbreaks. Information in this database comes from CDC, books, city and state health department files, news reports, and Public Health Service Regional files. Thus, the NETSU database is more complete but at the same time less precise. The NETSU data includes individual cases that were not reported as outbreaks. The CDC defines outbreak as two or more persons becoming ill after consuming a common food at the same time. Illness that only affects one individual will not be reported in the CDC database. For example, the NETSU database lists over one hundred thirty five cases of *Vibrio vulnificus*, but because this bacterium only affects individuals in high-risk categories, no outbreaks (two or more individuals having a similar illness after consuming the same food) have been reported. Thus, illnesses from this organism do not appear in the CDC database used for annual summaries.

The information in the tables is useful for estimating the risk from microbial contaminants. Most of the illness associated with molluscan shellfish has been associated with either enteric viruses or naturally occurring marine organisms of the family *Vibrionaceae*. Other microorganisms account for far fewer illnesses.

Hackney, C. (1993a) *The Molluscan Shellfish Industry*

The objective of this report was to provide an overview of the molluscan shellfish species that are harvested and the methods used to process the shellfish for market. The report is divided into two parts: shellfish species and processing.

Hackney, C. (1993b) *The Current Shellfish Indicator System: Opinion Survey of the Molluscan Shellfish Industry*

The purpose of this report was to determine what the molluscan shellfish industry believed about the current indicator system. Although 45 percent of the respondents believed that the current standards are too restrictive, 55 percent believed that the standards are not adequately protecting consumers. Only 27 percent believed that the current standards accurately reflect health hazards. Over-

all, the responses indicate that there are strong opinions on the standards and reservations about their effectiveness.

Kator, H., editor (1994) *Manual of Procedures for Analysis of Molluscan Shellfish and Growing Waters*

This manual, a compendium of existing methods, was developed to assist in the evaluation of shellfish and their growing waters. During the formulation of the strategy for the NIS, agreement on laboratory methods was determined to be a critical factor. The comparison and reliability of data gathered to identify, isolate, and enumerate both traditional and new indicators of public health risk associated with fecal contamination of molluscan shellfish harvest areas were believed to be highly dependent on the laboratory methods used and uniformity in the application of methods. Without reliable, comparable data, risk assessment across the field sites in the epidemiological investigation would not be possible. The purpose of this manual was to assemble under one cover a variety of methods for microbial indicators identified by early NIS committees and workshops as promising for additional study or for use in the NIS study. This compilation of methods was not intended to replace the highly detailed methodologies in the American Public Health Association, the Association of Analytical Chemists of the U.S., or the U.S. Food and Drug Administration manuals. The manual was more properly viewed as a compendium of many methods with potential application to the microbiological assessment of shellfish and their growing waters. Future manuals, which were not prepared because of termination of funding for the NIS, were envisioned as being considerably more detailed and containing those methods actually used in the NIS program.

Leonard, D., M. Broutman, and K. Caverly. (1988) *The National Collaborative Shellfish Pollution Indicator Site Selection* and Leonard, D., and E. Slaughter. (1990) *The National Collaborative Shellfish Pollution Indicator Site Selection: Phase II*

The third component of the NIS was an epidemiological study that was to evaluate the relationship between illness in volunteers ingesting raw shellfish in an intensively monitored and controlled situation, and the water quality in the harvest areas as measured by traditional and new indicators of fecal pollution. Recognizing the need to begin investigation and characterization of potential sites for the NIS, the program commissioned a review of available information in 1987 through 1988. Criteria for site selection were established. Recommendations were solicited from representatives of state and federal regulatory agencies and seafood harvesters and processors in five different regions of the United States. Preliminary selection of one hundred sites was made. This report describes the process used and lists, by region, the potential sites identified. The information contained in the 1988 report was reevaluated using a relational database program designed by EG&G Washington Analytical Services Center, Inc. and the National Oceanic and Atmospheric Administration. This approach reduced the number of sites for consideration to forty-six. The results of this analysis are contained in the report.

Leonard, D. (1992a) *Site Assessment Guidance Document*

The site assessment guidance document was written to facilitate the performance of site assessments at every site considered for inclusion in the NIS epidemiological phase. Ideally the work would be performed within the year preceding the beginning of controlled feeding studies. Although several subcontractors could be involved in a site assessment, all activities would be required to follow the procedural guidelines and quality assurance, as outlined in the manual. Data entry forms approved for the NIS were included in the appendices and would be required for all data collection and data submission. Whether data is entered into the computer by the on-site contractors or forwarded to the data management center for data entry, the formats used to receive the data would be those approved by the data management center. At the end of the site assessment process, the contractor was required to provide background information, hydrographic study results, and microbiological sampling results. Recommendations would then be made on sample site locations for use in the epidemiological phase.

Other NIS Products

The NISC issued a "white paper" on the National Indicator Study in June 1993. The report described the NIS approach to improving the water quality management system currently used to ensure the safety of raw molluscan shellfish. A summary of the three major components of the study, laboratory methods development and validation, field trial of methods at defined sampling sites, and a human feeding study to determine exposure–response relationships, was also provided in the report. Subsequently, in October 1993, a draft 5-year plan for the NIS was prepared by NOAA in cooperation with ISSC, NISC, and LUMCON. The plan elaborated on the white paper and included a final step to conduct a risk assessment and develop a risk management strategy. This long-range planning document was followed by the preparation of a Plan of Operations that was received and accepted in draft form by the NISC at its February 1994 meeting. All work on the planning documents was halted following the decision to discontinue the study.

IMPEDIMENTS TO COMPLETION

There are many reasons why the NIS was not completed as planned: political problems, issues with epidemiological studies, industry impacts and concerns and, the most critical, sufficient resources. Without a sufficient funding base, this scientifically based program could not be undertaken. In the initial scoping document prepared in Cocodrie in 1987, the team estimated that 25 million dollars would be sufficient and achievable with a special appropriation from Congress. Although Congress was supportive of the needs of the shellfish industry and the consumer public, they were not ready to commit to this figure. Thus, the funding was budgeted through NOAA in small amounts, sufficient to accomplish 10 research projects, but not sufficient to field test indicators and methods nor conduct a full-scale feeding study.

The political pressure to conduct a national-scale project to test indicators and develop new methodology came from the shellfish industry. Public officials, the scientific community, and consumer groups did not put their collective power behind the NIS, a move that might have guaranteed success. In fact, there was opposition in some organizations based on jealousies, fear of stepping into unknown territory, and unwillingness to change current systems. Politics within the scientific community created a barrier to full cooperation among the researchers. In order to ensure a successful project on an international level there will need to be full cooperation, trust, and transparency in most project-related activities.

The conduct of a full-scale feeding study is extremely complex

and requires the expertise of organizations and medical personnel who have successfully complied with all requirements in previous studies. Experimental subjects are well protected and often are well-paid volunteers. Follow-up can be lengthy, particularly in cases where a disease agent has been identified. However, as difficult and expensive as these studies may be, it is acknowledged that if we are to adopt a suite of indicators that are scientifically supported, the studies must be done.

The motivation to develop new indicators came from the shellfish industry. Their concerns were twofold; first, that shellfish-growing areas are closed based on a coliform indicator that is not indicative of human disease potential, and second, that conversely, some areas remain open to harvest although there may be pathogens of concern present in the shellfish. In the early 1990s, the industry had to change their focus from the NIS to illness and deaths occurring from *Vibrio vulnificus*, *Vibrio cholerae* non-01, and *Vibrio parahaemolyticus*. Even though the cases are few, the effects from *V. vulnificus* in immunocompromised consumers are often fatal. Under pressure from consumer groups and the press, the ISSC and shellfish industry have had to focus all possible resources on this issue.

U.S. EPA's BEACH Program

Within the United States as well as internationally, there are debates between agencies regarding the effectiveness of chosen indicators. For example, the U.S. EPA has been increasingly concerned with the public health risks of swimmers exposed to infectious diseases on our nation's beaches and the effectiveness of the indicators used to monitor swimmer safety. To counteract this problem, the EPA has established the Beaches Environmental Assessment Closure and Health (BEACH) Program. In 1986, the EPA issued a revision to its bacteriological ambient water quality criteria recommendations to include new indicator bacteria, *E. coli* and enterococci, which provide a better correlation with swimming-associated gastrointestinal illness than the previous criteria, fecal coliform bacteria. These revised criteria are useful to public health officials because they enable quantitative estimates of illness rates associated with swimming in polluted water. A new enterococci method is a revision of the EPA's previous method, used since 1985 in ambient water quality monitoring. It reduces analysis time to 24 hours and improves analytical quality. The method has been validated in single- and multi-laboratory studies and has undergone peer review.

IS AN INTERNATIONAL APPROACH FEASIBLE?

International Aspects

In the United States there are a number of federal agencies that are involved in the development and application of microbiological standards in food products including molluscan shellfish. These include the Food and Drug Administration, Department of Commerce, Department of Defense, Veterans Administration, Department of Agriculture, and Environmental Protection Agency. In addition, numerous state and municipal agencies and private firms are involved in application of these standards.

Much more complex is the array of international organizations with interests or roles in the discipline of microbiology in food regulatory systems. Among the fourteen identified organizations are FAO/WHO Codex Alimentarius Commission, European Economic Community(EEC), International Standards Organization, International Commission on Microbiological Specifications for Foods, Nordic Food Group, and the FAO/WHO-sponsored Expert Microbiological Consultations. In spite of the appearance of coordination, there is a mix of both microbiological and toxin standards applied worldwide (Leonard 1992b).

The major difference in microbiological standards used by the United States and other shellfish-producing countries is the application to waters rather than meats. Only the United States, Canada, and Chile classify shellfish-growing and -harvesting waters using the fecal coliform standard. On January 1, 1993, the EEC implemented health conditions for the production and placing in the market of live bivalve mollusks. Although there are some amendments, these standards have been adopted by all members. Chapter I of the Annex, Conditions for Production Areas, dictates how boundaries and classifications of production areas are determined. Despite these differences, the NSSP and EC Directive are based on the assumption that a relationship exists between sewage pollution of shellfish-growing areas and human disease.

Trade Issues

The United States has a bilateral agreement with Canada and FDA-approved programs in Chile, Korea, and New Zealand. However, trade between the United States and all other shellfish-producing countries requires certification by local authorities. This certification is based on an understanding or agreement that the standards in both countries are equivalent. Unfortunately, neither the industry nor the officials involved in the certifications feel confident that true equivalency exists. In fact, in some other situations, the U.S. industry believes that depuration or postharvest processing are unnecessary requirements although required of all imports to the receiving country.

Other issues shared by all shellfish-producing countries are risk assessment, management, and communication as well as HACCP and other inspection issues. These are areas in which we can work on a cooperative basis, focusing resources, exchanging ideas and technology, and building open communication and trust. If the guidelines and regulations are developed jointly, then the goals of transparency and equivalency are accomplished.

CONCLUSION

Based on discussions that took place at the International Conference on Molluscan Shellfish Safety at Southampton, New York, in June 2000, I believe it is not only feasible but also incumbent upon us to develop an International Working Group on Microbiological Standards and begin a process to develop better risk management tools. We are prepared to develop a web site based at the University of Florida that will open the doors to communication. The purpose of the site is to allow posting of new methods, findings, and shared ideas and concepts. It will also have the capability of conferencing and chat rooms. Rather than develop another layer of international bureaucracy, we will work scientist to scientist and manager to manager, involving the shellfish industry in our deliberations and information dissemination.

The extensive work that has been completed under the National Indicator Study will be available to everyone who can access a web site. In the interim, information can be accessed by calling the Interstate Shellfish Sanitation Conference at (803) 788-7559 or by accessing the web site at http://www.issc.org/issc/.

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THE USE OF HIGH HYDROSTATIC PRESSURE TO SHUCK OYSTERS AND EXTEND SHELF-LIFE

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ABSTRACT High hydrostatic pressure (HHP) was used for mechanically shucking oysters. Microbial, chemical and descriptive evaluations were performed over a 3–4 week period for HHP samples and controls. Results from this study demonstrated that HHP was effective in shucking oysters at 35,000 to 40,000 psi. Results showed that HHP treated oysters maintained higher pH than the control over the storage period. Moisture content of the control decreased about 3% while HHP treated samples increased 2%. Pressure treatment did not significantly inhibit lipase activity during the shelf-life study. It was observed that HHP had a significant effect on reducing initial microbial load by approximately 2 to 4 logs. Descriptive evaluation showed HHP treated oysters had better quality than that of control during the shelf-life period. HHP proved to be an effective method for shucking oysters and extending shelf-life.

KEY WORDS: hydrostatic pressure, shucking, extending shelf-life

INTRODUCTION

Oysters are filter feeders and contain a high level of microbial flora, which will cause eventual spoilage and may pose a threat to public health. There is an increasing concern with the safety of raw oyster consumption, but at the same time consumers demand remains high for oysters that retain the original nutrients, flavor and appearance. Consequently, high hydrostatic pressure (HHP) has become the newest and potentially most promising method to purify oysters. HHP is a 'heatless process,' which has an effect on microorganisms' survival and enzymatic activity in foods (Hoover et al. 1989). HHP induces a number of changes to morphology, biochemical reactions, genetic mechanisms and cell membranes and walls of microorganisms (Smelt 1998).

Previous work with HHP and shucked oysters has shown that this treatment is effective in reducing total bacterial load in shucked oysters (Shiu & Morrissey 1999). In addition, preliminary work with HHP and whole oysters indicated that the treatment could be used for mechanically shucking oysters. The high pressure appears to preferentially sever the adductor muscle from the shell without altering the appearance of the raw oyster. This combination of mechanical shucking and microbial reduction in oysters by HHP could dramatically change the traditional oyster processing operation. The objective of this study was to determine the optimum HHP conditions (pressure/time) for shucking the oysters and the effect of HHP on the microbial, biochemical and descriptive evaluation of oysters.

MATERIALS AND METHODS

Oyster Source

Live whole oysters and hand-shucked oyster meats used as controls were collected from a local oyster processor in Willapa Bay, WA, and transferred to the OSU Seafood Laboratory as soon as possible after harvest. Whole oysters were stored at 10°C during transportation, while shucked meats were stored at <4°C.

Pressure Treatment

Whole oysters were vacuum packed in a waterproofed bag and subjected to eight different pressure treatments: (a) Control samples—no treatment; (b) Pressure treatment 30,000 (30k) psi, 2 min; (c) 35k psi, 0 min; (d) 35k psi, 1 min; (e) 35k psi, 2 min; (f) 40k psi, 0 min; (g) 40k psi, 1 min; (h) 45k psi, 0 min. Samples were pressure treated in an isostatic pressure unit (Model IP-3-22-60, Autoclave Engineering, Inc.).

Sample Preparation

Shucked oysters, 4 from each treatment and controls, were packed with water into a 100 ml jar, 4 oysters per container. Before biochemical and microbial tests, oysters were aseptically transferred to a pre-sterilized blending jar. Samples were then homogenized for 3 s. Oysters used for sensory tests were directly transferred to petri dishes from jars.

Biochemical Tests

One gram of homogenized samples was diluted with distilled water (1:10) and tested for pH using a pH meter. Moisture content was determined by the oven drying method as described in AOAC (1984). Lipase activity was determined by the method based on detection of the fluorescence of 4-methylumbelliferone (4-MU) produced by enzymatic hydrolysis of substrates (Stead 1983).

Microbiological Tests

Aerobic plate counts (APC) were measured using petrifilm and all the plates were incubated at 25°C for 72 hours. Anaerobic plate counts (ANPC) were measured using the pour plate technique and were incubated in anaerobic chambers at 35°C for 48 hours.

Descriptive Tests

Descriptive tests were performed after HHP treatment by a trained panel using a freshness grade guide (developed at OSU Seafood Laboratory) determined over a three-week period. Quality Index Method (QIM) was used to predict shelf-life of all oyster samples (Bremner et al. 1985).

Statistical Analysis

Data were analyzed for significant differences using an analysis of variance (ANOVA). All statistical analysis was performed using STATGRAPHICS plus Version 3.1.

RESULTS AND DISCUSSION

Different HHP treatments had varied effects on degree of shucking, microbial counts and the descriptive evaluation of oysters' meat. HHP-shucking results showed that higher pressure and longer time caused optimum detaching of adductor muscle. The

minimum pressure/time parameters for approximately 90% success in oyster shucking (detachment of adductor muscle) was 35k psi for 2 min. All HHP treated samples maintained a pH above 5.7 for the shelf-life study, while the control sample decreased to 5.1 after 16 days of storage. Based on pH standards for oyster quality (Cook 1991), results from this study indicated that HHP treatment was able to extend the shelf-life of oysters up to 50%. Moisture results indicated that HHP treated oyster absorbed more water than the controls. HHP reduced initial microbial load and inhibited microbial growth during storage at refrigerated temperatures. After 14 days in storage the HHP samples were 2–4 logs lower in total aerobic counts and anaerobic count. In general, the higher pressures were more effective in lowering microbial counts. Descriptive tests showed that HHP treatment extended the shelf-life of oysters. All pressure treated oysters scored higher in descriptive evaluations than hand shucked oysters over the storage period. These results showed that pressures between 35,000–40,000 psi with a holding time of 1–2 min were effective in (a) shucking oysters, (b) reducing microbial count, and (c) causing minimal changes to the appearance of raw oysters. Although the effect of HHP on oysters may be dependent on species and seasonality, HHP is a promising method for shucking oysters and extending their shelf-life.

CONCLUSIONS

This study showed that HHP treatments were effective in producing safe, high quality oysters. In this study, a pressure treatment of 35k, for 2 minutes was optimum for shucking the oysters. Research will be continued to determine the effects of HHP on *Vibrio parahaemolyticus* inoculated into whole oysters.

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PRESSURES ON NEW JERSEY SHELLFISH WATERS

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INTRODUCTION

Since 1978, the State of New Jersey has been able to restore more than 90,000 acres of shellfish waters to harvestable status. These restoration efforts have resulted in classifying approximately 88% of the shellfish waters in the state as harvestable. (Harvestable waters include all shellfish waters from which shellfish are available for either direct market or market after additional purification, such as depuration.) Therefore, the state's goal to classify 90% of the waters as harvestable by 2005 is close to being achieved (Fig. 1). Additional waters have improved to a less restricted harvesting status. Most of the improvement in water quality prior to 1995 was directly related to improvements in domestic waste treatment. Evaluations of shellfish waters may be found at www.state.nj.us/dep/watershedmgt/bmw.

New Jersey is a densely populated state with a highly developed waterfront area. Much of the infrastructure, particularly in the northern coastal areas, has been in place for many years. As a result, existing pressures on water quality in shellfish waters are comprised primarily of non-point sources and unpermitted discharges.

POINT SOURCE PRESSURES

Point sources of domestic waste from treatment facilities have been consolidated into regional treatment facilities. There are no longer any permitted domestic waste point sources located in the back bay areas; all discharges are located in the deeper waters of the Atlantic Ocean. Consequently, the pressure on shellfish waters from these sources has diminished.

Pressures from unpermitted point sources, primarily spills, continue (Fig. 2). Although there are numerous unpermitted discharges into saline waters each year, most are insignificant. However, during the period between 1995 and 1999, nine unpermitted discharges resulted in suspension of harvest. Each resulted from a discharge of raw or partially treated domestic waste. The Bureau of Marine Water Monitoring routinely responds to situations in which sewage or other pollutants have been discharged into saline waters. Most of these discharges are the result of excessive inflow into collection systems during storm events or breaks in lines carrying waste materials. In some cases such discharges result in suspension of shellfish harvest in the affected waters.

NON-POINT SOURCE PRESSURES

Non-point source pressures on shellfish beds in New Jersey originate in materials that enter the water via storm water. These materials include bacteria as well as other waste that enters the storm water collection system.

The Bureau of Marine Water Monitoring has identified areas where the bacteriological water quality is adversely affected by storm water and has begun to identify particular storm water outfalls that discharge excessive bacteriological loads during storm events (Fig. 3). Areas were identified by comparing fecal coliform levels measured after significant rainfall with those measured after a period of dry weather.

In some cases, specific discharge points can be identified, as in the example of Seaside Heights, New Jersey, shown in Figures 4–6. In this case, a small area in Barnegat Bay adjacent to Seaside Heights was identified as a storm water–affected area, as described above. The area was surveyed to determine potential sources, including locating storm water outfalls, marinas, and areas of dense

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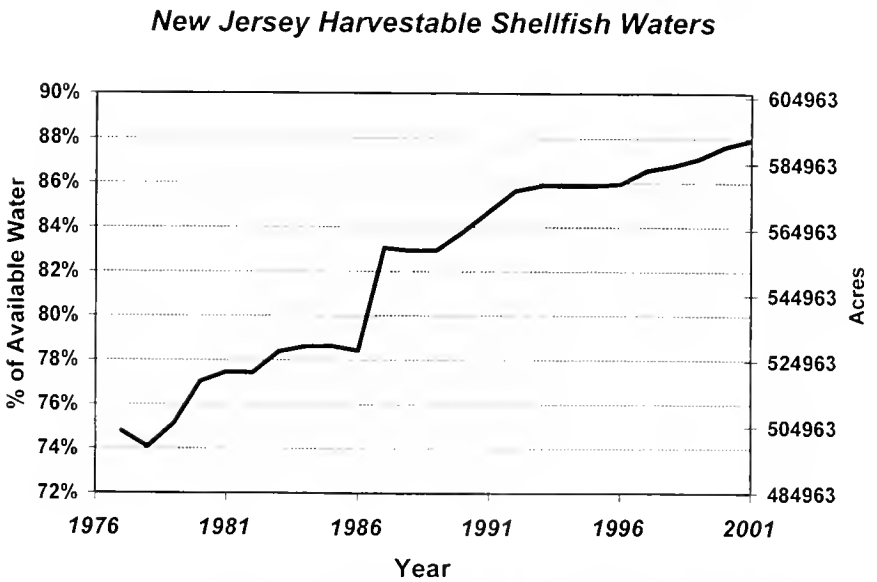

Figure 1. Percentage of total shellfish water in New Jersey classified as harvestable.

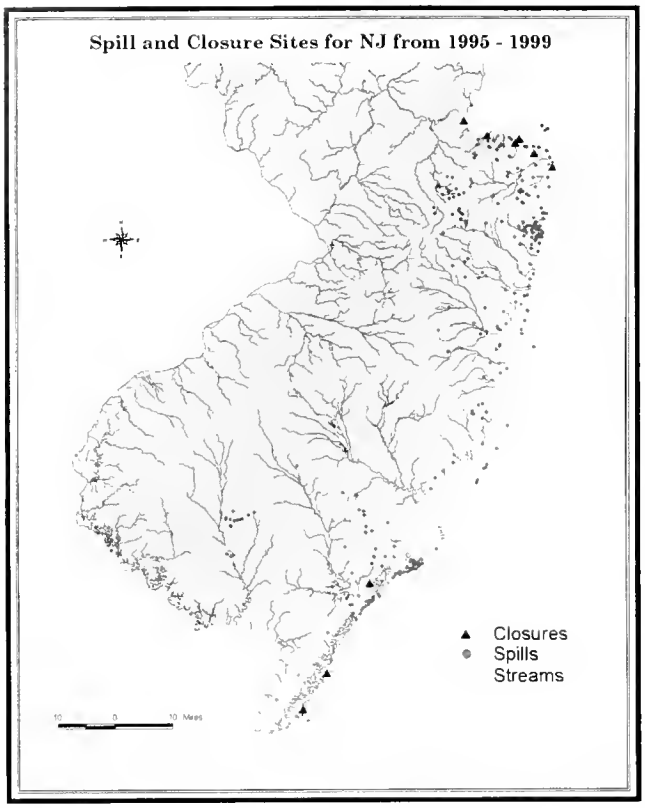

Figure 2. Spill and closure sites (1995-1999).

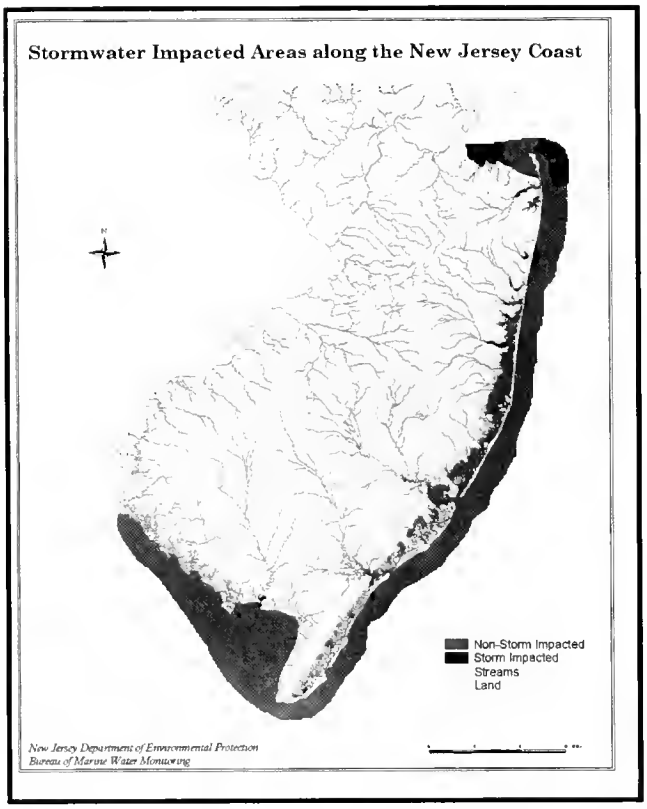

Figure 3. Storm water–affected areas along the New Jersey coast. Specific areas have been identified as those affected by storm events. These areas are being investigated further to determine specific sources of contamination.

Figure 4. Seaside Heights, NJ, concentration of *E. coli* prior to onset of precipitation.

Figure 5. Seaside Heights, NJ, concentration of *E. coli* 1 h after onset of precipitation.

Figure 6. Seaside Heights, NJ, concentration of *E. coli* 3 h after onset of precipitation.

wildlife populations. Samples were then analyzed during a significant storm event for *Escherichia coli* to further pinpoint the specific sources of bacterial contamination. Two storm water outfalls (of the 18 located in the area), where *E. coli* concentrations were >1,400 cfu/100 ml, were identified as probable sources (Fig. 5). Three hours after onset of precipitation, counts greater that 1,400 CFU/100 ml were measured in the northeast corner of the cove (Fig. 6). During the previous 2 h, strong winds from the southwest and incoming tide (entering under the bridge shown at the bottom of the map) affected the distribution of bacteria in the cove.

When specific outfalls are identified as significant sources, the Department of Environmental Protection works with the county and municipality to further refine the source(s) of the contamination and implement remediation activities. Next steps involve identifying the drainage area of the specific outfalls and then monitoring within the drainage area to identify the actual source(s) of the bacterial contamination. Once these have been identified, the state and local governments work to correct the problem.

CONCLUSIONS

Because it is often difficult to identify specific non-point sources of contamination, remediation of non-point pollution can become costly. This project demonstrates that a well-designed plan for sampling and analysis can help target resources to the specific areas causing elevated bacterial levels. Approximately 10% of the storm water outfalls in the area contribute significantly to the water quality impairment. Additional work is planned to differentiate between human and nonhuman sources (coliphage analyses) as well as to identify actual sources within each affected catchment area.

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SCIENCE, FOOD SAFETY REGULATIONS AND EUROPEAN SHELLFISH CULTIVATION

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Across Europe, shellfish farms are generally located in relatively unpolluted waters in peripheral regions, far from the madding crowds and their inevitable discharges to the environment. And across the European Union the shellfish cultivation sector promotes its natural, "organic" products as cultured in an environmentally friendly and sustainable manner. Yet, in recent years not only has the sector felt that it has been subject to excessive hygiene regulations, but consumers have been bombarded with images of a food product that is indistinguishable from the more intensively farmed species such as poultry, cattle, sheep, and salmon. Underlying this dichotomy and giving legitimacy to "food scare" stories is the body of science that identifies valid food safety concerns but fails to adequately define the risk factors involved and in effect contributes to the current climate of food hysteria.

Production of cultivated shellfish in Europe (1997) totalled some 665 thousand tons (KT), a volume dominated by mussels, which, at 515 KT, represented about three quarters of European output and around 70% of global production. Oyster production (*Ostrea edulis* and *Crassostrea gigas*) reached some 100 KT, 15% of total EU cultivated shellfish output but representing only 3% of world volumes. The third group of shellfish assessed, clams, accounted for around 50 KT, some 8% of European shellfish output and 4% of global production. With a first sale value of ~580 million euro (ME), shellfish is worth around 30% of overall European aquaculture. Although still the dominant species, in value terms the importance of mussels declined to 47% of the total, at some 270 ME, whereas first sale values for oysters and clams totalled around 150 ME for each species (26–28%). Average prices ranged from just over 500 E/ton for mussels to 3,000 E/ton for clams.

There is an equally skewed distribution of the location of activity across the Union. Shellfish production is geographically extremely concentrated, with 73% attributable to only three countries—Spain, the leader, with 198 KT or 30% of the total, followed by Italy with 143 KT (22%) and France at 141 KT (21%). In addition to these production and value parameters, a further particularly significant attribute of shellfish farming, reflecting the relatively "low tech" nature of operations, is the contribution it makes to employment opportunities. The sector is calculated to generate around 20,000 full time equivalent jobs in the production segment alone, over 50% of total direct employment in the European aquaculture industry.

Turning to food safety legislation, the initial thrust of EU-based food safety legislation took place during the late 1970s and early 1980s, when almost fifty directives relating to water and food quality and associated issues passed into national legislation. A total cost of $70 billion over the past 15 years for the UK alone has been estimated for the implementation of these directives. The main drivers for these and subsequent directives have been the need for harmonization of regulations in the context of the "single market" and the aim to improve food safety. In addition, there have been a number of high-profile food quality–related concerns—the European list is extensive, including BSE (beef), dioxins (poultry), listeria (milk, cheese), salmonella (eggs), *Escherichia coli* 0157 (meat products), Chernobyl fission products (lamb, mutton), GMOs/transgenics, residues (growth promoters, antibiotics, pesticides, heavy metals, hydrocarbons [PAHs]), etc.

The harmonization of specific shellfish food safety legislation was introduced in 1991, with two related directives, 91/492 and 91/493. The latter covers "fisheries products," whereas the former has the greater relevance for molluscan shellfish farmers because it focuses on "laying down the health conditions for the production and the placing on the market of live bivalve molluscs." The main objective for the directive, including later amendments, is to protect human health through the establishment of common requirements for harvesting, handling, storing, transportation, and distribution of molluscs, including permitted maximum levels of bacteria and biotoxins, classification of harvesting areas, and authorization of depuration centers. The fundamental objectives of 91/492 and the other food standard directives, namely, to improve the quality and safety of food, are applauded by the industry, in principle, because shellfish farmers recognize the paramount importance of minimizing the risk to human health from their products and have always been aware of public concerns over shellfish. They also recognize the positive benefits of harmonized standards across a single market of 250+ million potential consumers and that higher hygiene standards will improve the product image with the public. Indeed, any reduction in reported or alleged shellfish "poisoning" stories in the media is always gratefully welcomed by the industry, which believes its products are too often unfairly blamed for consumers' overindulgence, not necessarily of shellfish! Nevertheless, the industry also has some significant concerns about both the legislation itself and the manner of its implementation, and a number of legitimate queries have been voiced. Other industry criticisms about the details of the legislation include complaints about paper trails that don't work, water recycling criteria without scientific justification, the use of "old fashioned" bacterial indicators for the viral problem, and the continued reliance on the unfortunate "mouse" test.

The cost of compliance with Directive 91/492 has proved to be a major financial burden for the sector due to the extent and detail of the new requirements for harvesting equipment, depuration and dispatch facilities, and transportation units. Necessary capital expenditure alone over the first five years after introduction has been estimated to total some 350 ME, and ongoing incremental operating costs (personnel, equipment, facilities, overhead, documentation, testing, etc.) have been estimated at a minimum 30 ME per year.

In addition to these direct financial costs, there has been the indirect cost of lost output as investments into expanding production capacity were diverted into equipment and handling facilities while profit margins, required to finance future expansion programs, were slashed. Although clearly only an estimate, comparing potential expansion with actual volumes during the mid-1990s (minimal growth for oysters and clams, and a decline followed by slow recovery for mussels), the cumulative value of lost produc-

tion has been calculated at some 500 ME. Furthermore, lost production across the EU as a result of closures for apparent biotoxin contamination (PSP, DSP, ASP) above the Action Levels is estimated at an additional 50 ME for the period since 1992. In aggregate, these are not insubstantial sums, even for an industry with an annual turnover in 1997 of almost 600 ME!

Regular monitoring for biotoxins is a long-established practice in many European countries, as it is elsewhere; however, since the introduction of 91/492, the process has become more extensive and more regular while the techniques and protocols of the analyses have become more standardized, at least in theory. The network of national reference laboratories, combined with a central European Reference Laboratory (located at Vigo, Spain for this topic), enables scientists to tap into an extensive database of information and discuss in an informed manner the various issues of concern as expressed by food safety interests and the production sector.

Reported presence of PSP toxins in European shellfish over the past decade has ranged widely, from the Straits of Gibraltar to the North Cape, with a generalized perception that the incidence is becoming both more intense (higher levels of toxin) and more frequent. But whether this is a long-term trend or part of a cycle has not yet been determined; there are still many who insist on the latter, pointing out that biotoxin contamination is a natural phenomenon and the source of many cultural and religious regulations about shellfish consumption. The detection of DSP toxins has also been a regular and widespread event over the decade, although in comparison with PSP, there appears to this author to be a southern and western "shunt" in incidence. ASP is the "new kid on the block," and so inevitably there are more limited indications of presence, with reports limited essentially to Scotland and Spain (one positive from Denmark and two from Portugal).

With only limited closures for PSP and DSP (although a greater incidence of the latter than in previous years), 1999 was without doubt the "year of ASP," particularly in Scotland. There were extensive closures both there and in Ireland, significantly limited almost exclusively to scallops (*Pecten maximus*), which made it a bit of a Celtic event, although there were reports of ASP in Galicia, Spain. This particular biotoxin event has not been "just another closure," but has had a number of specific characteristics and has raised several novel issues:

- As mentioned earlier, the toxin accumulation was restricted almost exclusively to scallops;
- Both cultivation and capture fishery sectors were affected, compared with the historical situation where closures due to PSP and DSP have largely affected only the farmers, who currently have relatively limited economic and political weight;
- The geographical extent of the "event," extending the length of the western coast, from Cape Wrath to the southern border with Ulster waters, areas off the eastern coast, and the Northern Isles;
- The temporal extent, from May 1999 for some twelve months (albeit, eventually, over a diminishing area);
- The use of FEPA (Food and Environment Protection Act) Orders to close areas, including shellfish farms, in contrast to the more flexible Voluntary Closure Agreements, which had previously been the normal management tool;
- The creation of a climate of uncertainty and distrust, fostered by poor communication between authorities (including scientists) and industry, lack of clarity on the testing methods, a specific suspicion of the negative impact of salmon farming on plankton populations, and perceived lack of government and scientific interest in the plight of the scallop sector and by inference the marine environment.

As the closures continued, there were increasing demands for focused research into the cause of the ASP event (because if this was a fact of life, with endemic reoccurrence, then the industry needed to restructure swiftly).

As the closures spread, there was an ever more vocal call for a review of the management regime, which appeared, in the United Kingdom at least, to have only two settings—"go" and "total stop"! Other countries, most notably our close neighbor, Ireland, appeared to have discovered a middle way, whereby the fishery and farms could continue harvesting despite ASP analyses above the Action Level but only place shucked scallop meats on the market. This approach reflected the reported differential accumulation of toxin in the different parts of the scallop, with the majority to be found in the hepatopancreas and only trace amounts in the adductor muscle (the white meat). Indeed, the Association of Scottish Shellfish Growers carried out a small-scale research project to try to establish this differential accumulation in some of their own animals. The results showed that 100% of "total tissue" results were significantly > Action Level of 20 μg/g (site means ranging from 112 to 203 μg/g for 4 separate locations), whereas the hepatopancreas accumulated in excess of 95% of the toxin (ranging from 1,400 to 2,600 μg/g). However, for the edible body parts, only 25% of "gonad alone" results were > Action Level, while zero "adductor muscle alone" results were > Action Level (83% below 10% of Action Level), and zero "adductor plus gonad" results were > Action Level (75% below 10% of Action Level).

These results indicate that the threat to public health from the consumption of adductor and gonad together, from scallops with relatively high concentrations of ASP as measured by a total tissue analysis, was, in terms of consumer risk, essentially nonexistent. The theoretical public health risk was further reduced if consumption of only adductor muscle is assessed. As a result, the Scottish industry has been demanding a comprehensive review of the current monitoring, testing, and management regime, both in Brussels and in the United Kingdom.

Achieving this objective will require a major contribution from the scientific community. First, there must be a greater effort from scientists to communicate with industry, to explain and justify procedures and priorities; lack of transparency creates a sense of exclusion and distrust, which in turn leads to fear and hostility. Second, scientists must venture down from their apocryphal ivory towers and debate research priorities with industry, seeking to identify R&D that will support and assist industry rather than subject it to additional constraints; after all, without an industry to support, there would be less call for their scientific expertise! Third, scientists must evaluate realistic levels of risk to human health, transferring experimental laboratory results into the context of the real world and transforming them into pragmatic policy parameters.

CONCLUSIONS

In the real world, where people need income-generating activities (jobs), where all foods can never be, and indeed should never be, 100% risk free, where there is a need for a rational risk assessment seeking the optimal way forward for all sections of society, the demand for responsible science is urgent. Protection of consumers from marine biotoxins, without destroying the supply chain, through appropriate scientific research, the application of

reason, and pragmatic, risk assessment–based management systems should be a common objective of science and industry. The intervention of credible and comprehensive science is essential in order to overcome the paranoia of politicians and bureaucrats driven by post-BSE angst.

A real world shellfish hygiene management regime must incorporate both monitoring and risk assessment, with the twin objectives of safeguarding public health while enabling shellfish producers to continue to supply quality products to the marketplace. For example, for biotoxins specifically, such a system would display the following elements:

- *For monitoring purposes*, sampling of the water column for evidence of rising cell counts of potentially toxic phytoplankton and diatoms;
- *For monitoring purposes*, total tissue testing, to establish the presence of biotoxins in shellfish populations;
- *For food safety purposes*, testing of toxin levels in the gonad (highest toxin loading of the edible portions):

If < Action Level, marketing should continue of both the shucked meats (adductor and gonad) as well as whole, in-shell scallops with a health advice note (analogous to the currently required Health Mark) stating "Only the white meat and coral (adductor muscle and roe) of these animals should be consumed";

If > Action Level, sales of whole scallops should be suspended and sales of adductor muscle alone would be permitted from authorized processing facilities.

Such a system would prioritize the concern for safeguarding public health while addressing the "specific risk material" issue and targeting the testing regime on the product being placed on the market. There is a clear role for science in this situation to assist in creating and supporting such multifaceted solutions to problems.

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HARMFUL EFFECTS OF THE TWO PILATE MOLLUSCAN SPECIES *CYMATIUM PILEARE* AND *LINATELLA CAUDATA* ON THE CULTURE OF PEARL OYSTERS IN VUNG RO SEA WATER, PHU YEN, VIET NAM

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ABSTRACT *Cymatium pileare* and *Linatella caudata* are two injurious predators to the pearl oyster *Pinctada martensii* cultured in Vung Ro cove, Phu Yen Province, Viet Nam. Ten culture cages of *P. martensii* containing 150 individuals each were kept under observation. After nearly a year of observation, the initial number of oysters, 1,500, was decimated by the two predators and reduced to only 67. Today, there are no better methods of destroying *C. pileare* and *L. caudata* than pulling the cages to the surface once every ten days to get rid of the predatory shells.

The pilates *Cymatium pileare* (Linne, 1758) and *Linatella caudata* (Gmelin, 1791) both belong to the family Cymatiidae, the superfamily Cymatiacea, the order Mesogastropoda, and the class Gastropoda. The species *C. pileare* (Linne, 1758) is fairly widely distributed and characterized by certain secondary varying features, assuming such forms as *C. pileare* from *aquatile* (Reeve, 1844) and *C. pileare* from *intermedium* (Pease, 1869). The species *L. caudata* has synonyms of *Linatella cingulata* (Lamarck, 1822) and *Linatella cutacea* (Lamarck, 1816).

The shell of *C. pileare* is big, heavy, lozenge shaped and oval (Fig. 1). *C. pileare* is widely distributed in the coastal waters of the Indian Ocean, Pacific Ocean, and other expanses in the Atlantic Ocean. The distribution is also reported in Vietnam's coastal seawaters. The carnivorous creatures attach themselves to sublittoral gravels at depths of 2–3 m. At an early stage, the shell cortex develops into a pile, making the body light enough to be attached to floating objects. At Vung Ro, *C. pileare* in pearl oyster cages ranged in size from 18–59 mm (height) and 10–31 mm (breadth) (Table 1).

Shells of *L. caudata* are of moderate size, and the body base abnormally bulges outward (Fig. 2). Like *C. pileare*, *L. caudata* is widely distributed from the coastal waters of the Pacific Ocean, the Indian Ocean to the Atlantic Ocean, and South Africa. The distribution is also reported in Vietnam's coastal seawaters. Its distribution according to water depths and substrata is similar to that of *C. pileare*. At an early stage, the shell cortex develops into a pile, enabling the creature to attach itself to floating objects. The species is carnivorous. Table 2 shows the ranges in size for *L. caudata* in pearl oyster cages at Vung Ro.

TABLE 1.

***Cymatium pileare* sizes in pearl oyster *P. martensii* culture cages at Vung Ro on April 21, 2000.**

Order	Height (mm)	Breadth (mm)	Height-to-Breadth Proportion
1	18	10	1.80
2	39	21	1.85
3	40.5	20.5	1.97
4	42.5	24	1.77
5	44.5	26	1.71
6	52	29.5	1.76
7	57	29	1.96
8	58	32	1.78
9	59	31	1.90

We observed that the floating larvae reaching the spat stage attached themselves to the shells of the growing oysters. The radulae and mandibles gradually took shape. The creatures, attached to the viscera of the oysters, gnawed away the oysters' flesh with their radulae and mandibles, causing the oysters' death. As a result, the number of oyster decreased in cages with increasing numbers of snails.

Ten rectangular pernio-netting cages were used to determine harmful effects of *C. pileare* and *L. caudata* on cultured pearl oysters. Each cage contained 150 seed oysters ranging in size from 2–3 cm. Inspections were conducted twice each month and involved destroying the snails in the cages and counting the dead

Figure 1. ***Cymatium pileare*** **shells.**

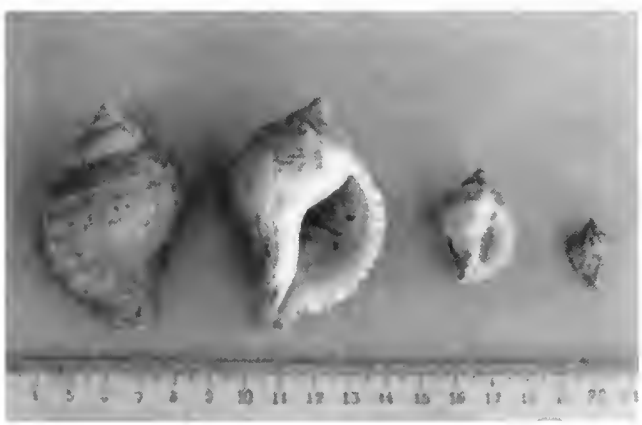

Figure 2. ***Linatella caudata*** **shells.**

TABLE 2.

Size of *Linatella caudata* in pearl culture cages at Vung Ro on April 21, 2000.

Order	Height (mm)	Breadth (mm)	Height-to-Breadth Proportion
1	16.5	10.5	1.57
2	19	11.5	1.65
3	20	12	1.66
4	23	14	1.64
5	25.5	15.5	1.64
6	32	19	1.68
7	35.5	21	1.69
8	39	24.5	1.59
9	42.5	26	1.61
10	46	29	1.58
11	48	29	1.65
12	61	37	1.64

oysters. Temperature and salinity were kept under strict observation during inspections.

After nearly a year of study, it was observed that the initial 150 pearl oysters cultured in each of 10 cages was reduced to only 38 in cage No 5 and 29 in cage No 6. The total number of remaining live oysters was 67, 1,433 having died. If 30% of the oysters (430 oysters) died from natural conditions and weakened resistance, then those oysters which were destroyed for food by *C. pileare* and *L. caudata* would number 1,003 (Table 3). *C. pileare* and *L. caudata* are therefore a great danger to the culture of the pearl oyster *P. martenssi*.

Observing oysters for the appearance of shells of the two pilates over a period from January to November 1999, we found that the two species appeared in the same seasons. In the pearl oyster cages, *C. pileare* accounted for 55–60% and *L. caudata* accounted for 40–45%, with more shells in cages at a water depth of 5 m than in those at 2 m. In March, young shells appeared in large numbers and grew quickly. They also appeared in June. This suggests that *C. pileare* and *L. caudata* reproduce from February to March and from May to June. From July on, no offspring were seen. If cages are checked every half month to remove existing pilate shells, only 1 or 2 shells can be found in the cage the next check time, but if shell-removing times are 30–45 days apart, 3 and sometime 7–8 shells are found in cages.

Currently, there is no better method for getting rid of *C. pileare* and *L. caudata* than pulling cages out of the water and destroying the shells every 10 days. Alternative strategies like chemicals or modified salinity would negatively affect the growth of *P. martensii* in the cages.

TABLE 3.

The effect of *C. pileare* and *L. caudata* on *P. martensii* survival in cages. Oysters were introduced into cages on December 15, 1998.

Check Date	No.	Number of Oysters	Number of Dead Oysters	Snails in Cages	Notes
31/1/1999	1	144	6	2	S‰:34
	2	145	5	1	T°C:29
	3	145	5	2	
	4	148	2	1	
	5	145	5	2	
	6	147	3	1	
	7	145	5	2	
	8	145	5	1	
	9	143	7	2	
	10	145	5	2	
	1–10	1452	48	16	
12/2/1999	1–10	1358	95	17	S‰:34 T°C:28
22/2/1999	1–10	1285	63	15	S‰:34 T°C:23
04/3/1999	1–10	1260	41	15	S‰:34 T°C:24
20/3/1999	1–10	1121	72	13	S‰:35 T°C:27
5/4/1999	1–10	947	114	13	S‰:35 T°C:25
22/4/1999	1–10	845	102	13	S‰:35 T°C:27
8/5/1999	1–10	727	118	13	S‰:32 T°C:26
26/5/1999	1–10	624	103	13	S‰:34 T°C:28.5
16/6/1999	1–10	491	133	13	S‰:32 T°C:28.5
6/7/1999	1–10	369	122	13	S‰:34 T°C:28
26/7/1999	1–10	311	58	10	S‰:33 T°C:27
16/8/1999	1–10	198	113	11	S‰:34 T°C:28
6/9/1999	1–10	135	63	12	S‰:32 T°C:26
26/9/1999	1–10	101	36	10	S‰:34 T°C:27
16/10/1999	1–10	79	12	11	S‰:35 T°C:28
6/11/1999	1–10	67	12	11	S‰:35 T°C:28

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EFFECT OF CHLORINE ON DIFFERENT BACTERIAL GROUPS AND EFFECTIVENESS OF THE PURIFICATION OF SEWAGE IN HARVESTED AREAS

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ABSTRACT Besides microbiological monitoring programs, it is important in shellfish harvesting areas to understand the process of purification of sewage discharged in harvesting areas. The objective of this study was to evaluate the importance of purification, the effectiveness of chlorination, and the influence in these areas. Chlorine produced significant reductions on all studied bacterial groups and reductions were among one and two orders of magnitude, being lower for fecal streptococci and aerobic bacteria at 25°C. High quantitative variability was observed with regard to the number of bacteria presents in these waters (W1, W2, W3) but qualitative variability is unknown. Correlation among shellfish samples and W3 (effluent discharged in harvesting areas) was not observed.

KEY WORDS: chlorine, purification, microorganisms, shellfish, microbiological classification

INTRODUCTION

Escherichia coli is the most commonly used indicator of fecal pollution and its presence in the environment is related to the presence of enteric disease (Rice et al. 1991). The EU Directive 91/492/ECC (Anon. 1991) classifies harvesting areas for bivalve molluses into categories A, B, C, and D depending on the level of fecal coliforms and *E. coli*. Harvesting areas from Galicia are divided into categories A, B, and C because along the 1195 km of Galician coast there are no areas where fecal coliform concentrations approach zone D values. A review of the microbiological classification is conducted once per year. The spatial and temporal frequency of sampling is higher than the European, Spanish, and regional regulation (Anon. 1991, Anon. 1993, Anon. 1999) requirements because Galicia is an important aquaculture region where 89% of the total aquaculture in Spain is located (Cruz Ferreiro & Noguera Mendez 1999).

MATERIALS AND METHODS

Between July 1998 and April 2000 three samples of water were analyzed every 15 days from a sewage farm located in Tragove (Cambados-Pontevedra, NW Spain) that concentrates 30,000 habitants. Water sampling sites were established as follow: Water 1 (W1) was untreated sewage; Water 2 (W2) was sewage after biological treatment and before chlorination; Water 3 (W3) was sewage after chlorination. These samples were collected between 9:00 AM and 11:30 AM, waiting approximately 40 min between collection for W2 and W3 to allow chlorine to act on bacteria. The volume of free chlorine was constant (0.1–0.2 mg L^{-1}). In the same period of time, wild mussel sampling occurred every 15 days at three sampling sites (San Sadurniño, Tragove, and Peña Mourelló). From July 1998 to July 1999, a sampling of mussels on rafts took place every 15 days at two locations (Cambados D and Grove A) (Fig. 1). Therefore, data are paired as follows: W2 and W3, to compare the effectiveness of chlorination; W3 and all samples from mussels, to determinate the influence on harvesting areas. W1 data allow the estimation of the concentration of different bacterial groups that arrive at this sewage farm, but this is not discussed in this paper.

Water samples were analyzed by membrane filtration (MF) technique (APHA 1995), except for five months due to equipment breakdown. During that time the multiple tube fermentation technique (APHA 1995) was used for total coliforms, fecal coliforms, and fecal streptococci and the 3M Petrifilm™ (Europe Laboratoires Santé, Cergy Pontoise, France) technique was used for aerobic bacteria cultured at 25°C and 37°C. When using MF, nutrient agar was used for aerobic bacteria at 25°C and 37°C, endo-B agar for total coliforms, m-FC agar for fecal coliforms, Slanetz & Bartley agar for fecal streptococci and TCBS (Thiosulfate Citrate Bile Sucrose) agar for *Vibrio sp.* Incubation times were three days for aerobic bacteria at 25°C, 48 h for aerobic bacteria at 37°C and fecal streptococci, and 24 h for fecal coliforms, total coliforms and *Vibrio sp.* When using multiple tube fermentation, a five tube and three dilution most probable number (MPN) was used. Brilliant green bile broth (BGBB) was inoculated with dilutions made from samples of water (W1, W2, W3), then incubated at 37°C for up to 48 h for total coliforms. Tubes showing gas and/or acid were examined for the presence of fecal coliforms (presence of gas) by subculture to BGBB, prior to incubation at 44.5°C for 24 h. This method was used for fecal streptococci but the medium was dextrose-azide broth (37°C, 48 h). Tubes showing sediment and/or turbidity were subcultured to ethyl violet azide (EVA), incubated at 37°C for 48 h, then examined for the presence of violet sediment (Plusquellec et al. 1983). Aerobic bacteria analyzed by 3M Petrifilm™ were incubated for three days at 25°C and two days at 37°C. One milliliter of the sample was inoculated in a thin special plate and the plate was incubated. The MPN technique was also used for enumerating bacteria in shellfish samples.

All counts are expressed as units for MF and MPN counts and were logarithmically transformed to normalize the variables for statistical analysis. Therefore, data for MF and MPN analyses were combined. Results were analyzed by variance analysis (ANOVA; $P = 0.05$) to determine if the chlorine significantly reduced the levels of the different microorganisms. However, these counts cannot be considered as absolute values because sometimes excess or the absence of growth made counting colonies impossible. To transform results as "higher than" or "not detected" in results suitable for statistical analysis, the following assumption was used: when the result was "higher than" the counting was obtained with the multiplication of the dilution factor by 500 (i.e., for a dilution factor of 10^{-3} this means 10^{-3} by 500), and when the result was "not detected" the counting was obtained with the multiplication of the dilution factor by 1 (i.e., for a dilution factor of 10^{-3} this means 10^{-3} by 1).

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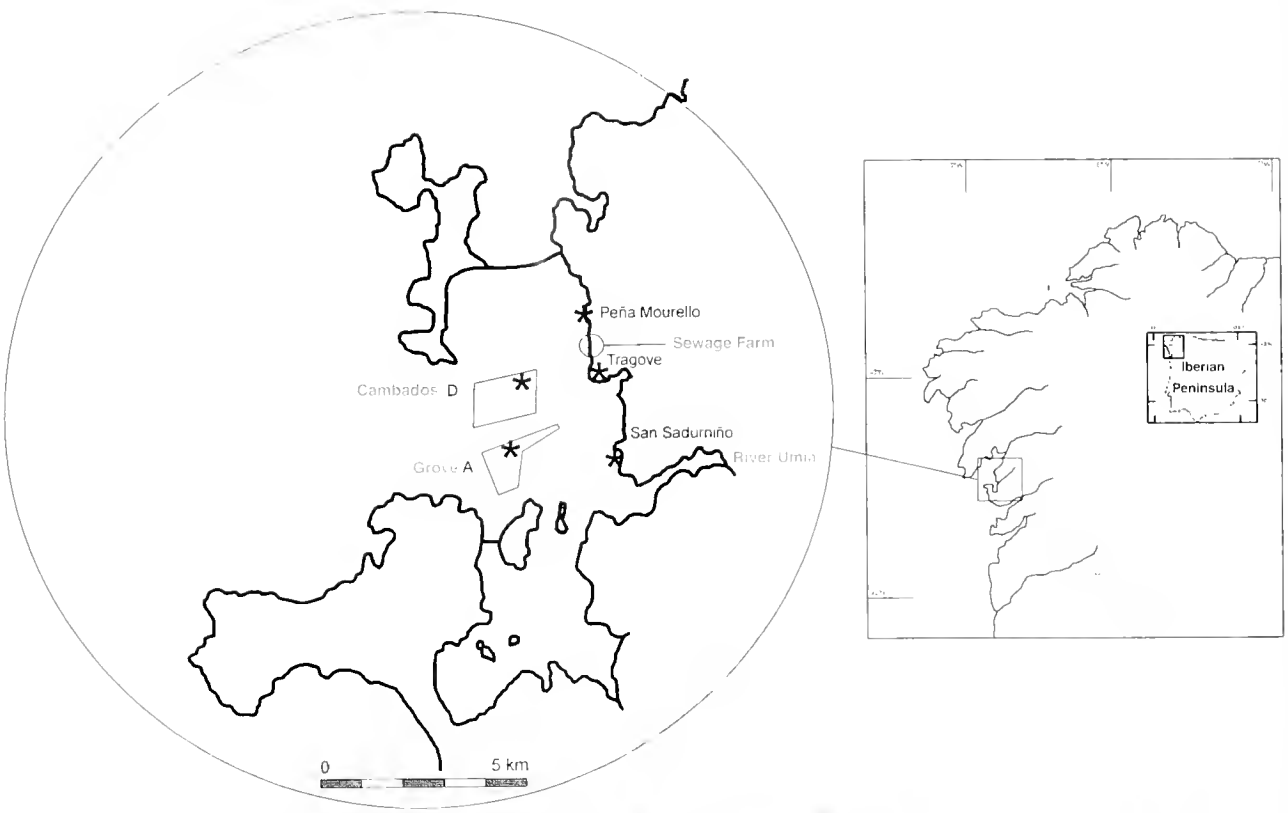

Figure 1. Sampling sites for bivalve molluscs. Cambados D, Grove A: sampling sites of mussel on rafts; Peña Mourelló, Tragove, San Sadurniño: sampling sites of wild mussel (bottom).

TABLE 1.

Average counts (per 100 mL) obtained for the three different types of sewage (W1, W2, W3).

Samples	Aerobic 25 C	Aerobic 37 C	Total Coliforms	Fecal Coliforms	Fecal Streptococci	Vibrio sp
W1	$550 \cdot 10^6$	$360 \cdot 10^6$	58,400,000	3,950,000	1,400,000	1,250,000
W2	39,700,000	10,500,000	2,900,000	680,000	93,000	90,000
W3	2,300,000	650,000	28,000	2,000	1,000	800

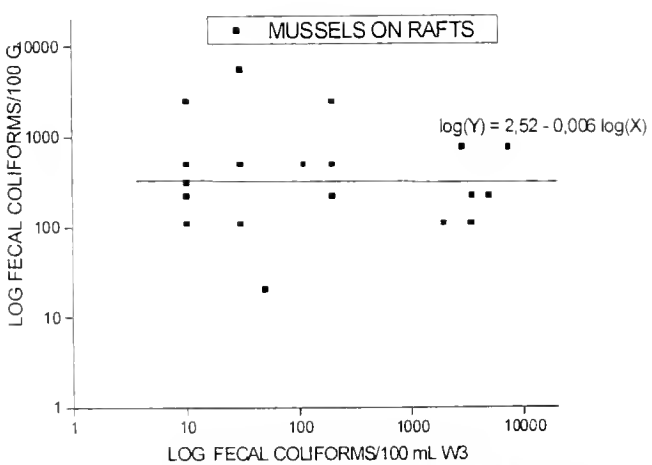

Figure 2. Correlation of log FC concentrations in mussels on rafts and W3 (R = 0.013).

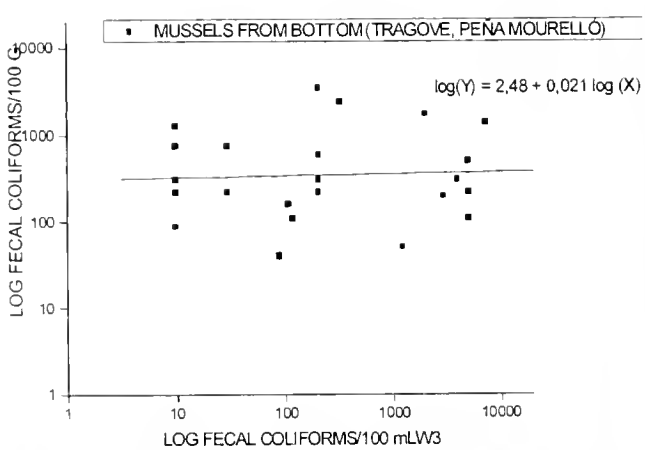

Figure 3. Correlation of log FC concentrations in two sampling sites mussel from bottom (Tragove, Peña Mourelló) and W3 (R = 0.045).

TABLE 2.

Standard deviation (per 100 mL) obtained for three different types of sewage (W1, W2, W3).

Samples	Aerobic 25 C	Aerobic 37 C	Total Coliforms	Fecal Coliforms	Fecal Streptococci	Vibrio sp
W1	$630 \cdot 10^6$	411,000,000	97,450,000	4,500,000	2,280,000	2,100,000
W2	$94 \cdot 10^6$	14,000,000	4,100,000	2,950,000	140,000	160,000
W3	9,350,000	2,200,000	52,000	2,200	1,980	1,800

RESULTS

Averages (Table 1) and standard deviations (Table 2) show values not logarithmically transformed. High variability was observed with regard to the number of microorganisms present in these waters. This variability could be explained by the fact that at the sewage farm in Tragove, bacteria enter by routes other than as feces; for example, bacteria enter the farm in rainwater and effluent from factories related to seafood and therefore environmental microorganisms can be detected in W1, W2 and W3.

Chlorine produced a significant reduction (ANOVA: $P = 0.05$) for all groups of microorganisms in effluent from the sewage farm (W3). These reductions were two orders of magnitude for bacteria at 37°C, total coliforms, fecal coliforms, *Vibrio sp.* (colonies grown on TCBS) and one order of magnitude for aerobic bacteria at 25°C and fecal streptococci. Lower reductions for fecal streptococci (Ritter & Treece 1948) may be explained by the fact that these bacteria adhere to each other in chains and clusters of various size and appear to be more resistant to disinfectants (Berg 1978). With regard to aerobic bacteria at 25°C, they comprise a heterogeneous group wherein the resistance to disinfectants is very different (Haas & Engelbretch 1980). For all waters (W1, W2, W3) a decrease in the number of bacteria was observed when aerobic bacteria at 37°C, total coliforms, fecal coliforms, fecal streptococci and colonies grown on TCBS were considered sequentially (Table 1). Lower counts of total coliforms than aerobic bacteria (37°C) are expected because mesophilic microorganisms comprise a much larger number of organisms than that constituted by total coliforms, which are defined as those that catabolize lactose with the formation of acid and gas (APHA 1995) at 37°C. Fecal coliforms are as subgroup of total coliforms. The higher number of fecal coliforms than fecal streptococci is explained because fecal coliforms, generally, are more abundant in human feces (Peres et al. 1980).

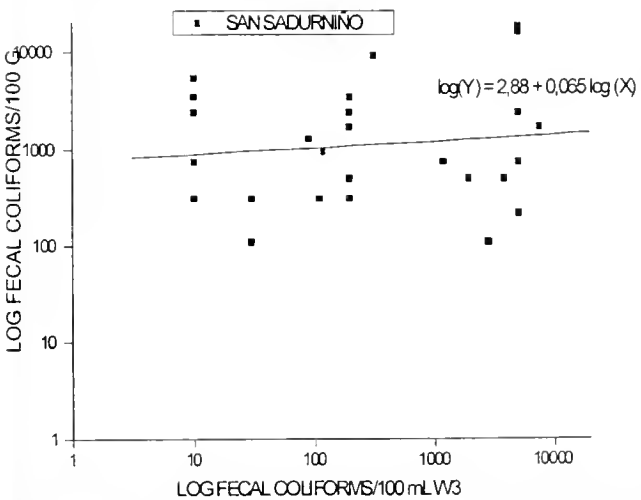

Figure 4. Correlation of log FC concentrations in one sampling site mussel from bottom (San Sadurniño) and W3 (R = 0.018).

Some species were isolated from colonies on the various agar media, most of them *E. coli* (eosine methylene blue agar Levine), *Klebsiella pneumoniae* (eosine methylene blue agar Levine), *Aeromonas hydrophila* (TCBS agar) and *Vibrio alginolyticus* (TCBS agar), but other isolates were not possible to identify. The number of sucrose-positive colonies was higher than the number of sucrose-negative colonies on TCBS. Counts of bacteria on TCBS were referred to as "microorganisms grown on TCBS" because it was not possible to identify some of them (all were oxidase-positive). Bacterial groups were identified using API 20E and API STREPT (Biomerieux, Maercy l'Etoile, France).

DISCUSSION

shellfish were used to establish a comparison with fecal coliforms in W3, even though in Galicia harvesting area microbiological classification is based on *E. coli*. In all sampling sites, except San Sadurniño, no significant differences (ANOVA: $P = 0.05$) have been observed for fecal coliform concentrations in shellfish and fecal coliform concentrations in W3. Correlation for this parameter in sampling sites and W3 was not observed (Fig. 2: R = 0.013; Fig. 3: R = 0.045; Fig. 4: R = 0.118), but it is possible that the high flow of the effluent (6000 $m^3 L^{-1}$) contributes to the presence of fecal coliforms in the area. Obviously, the dynamic nature of marine waters (Rosón et al. 1996, Peres et al. 1980) probably would have a significant influence. The different behavior for San Sadurniño may be related to its proximity to the mouth of the River Umia (Fig. 1) and therefore other conditions could have an effect (i.e., fecal coliforms from villages near the river that are carried out by it).

ACKNOWLEDGMENTS

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THE USE OF NONGOVERNMENT SAMPLERS IN DELIVERING A GOVERNMENT-MANDATED SHELLFISH SANITATION PROGRAM

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INTRODUCTION

In the past, the shellfish sanitation programs in many areas of North America were adequately funded compared to the needs of the industry. To some extent, this was due to the localized nature of the industry, with government offices located nearby. In addition, the abundance of other fisheries caused in some locations shellfisheries to be relegated to seasonal work or employment by limited populations.

Today, after decades of downsizing or rightsizing, we are seeing a decrease in the ability of the government to provide basic services in all areas and to provide specialized services in some areas. Ken Georgetti (2000) writes, "When what are essentially public services are delivered by private industry, the onus is on industry to prove the safety of its products or services. Regulation requires stringent scientific testing to prove safety, lengthening the time it takes to get a product or service to market. Once there, it requires rigorous adherence to safety standards. This onus to ensure safety costs business money. Deregulation is about increasing the margin of profit by cutting the cost of doing business."

Compounding this is the increase in both public awareness in safe food and the use of the resource due to the decrease in stocks of other fisheries. The worldwide boom in aquaculture has allowed employment in rural areas at a time when services to rural centers have been curtailed. In addition, the recognition of the rights of other users, such as aboriginal and the public, has seen the shift in direction for the program away from a traditional wild harvest. These fisheries have in some cases seen shellfish consumed in the raw or lightly cooked state according to the influence of ethnic and traditional backgrounds. This increased demand has led to regional "band-aid" solutions. These ad hoc arrangements, while solving immediate needs, have led to the increased liability to a government due to the lack of a system of checks and balances.

CASE STUDIES

To address these concerns, a government must be proactive. In February 1992, the Fish Inspection Directorate of Fisheries and Oceans (DFO; now the Fish, Seafood and Production Division [FSPD] of the Canadian Food Inspection Agency) introduced a new and innovative approach to fish inspection called the Quality Management Program (QMP). The program was developed jointly through the cooperative efforts of the fish processing industry and DFO and is based on five of seven HACCP (hazard analysis and critical control point) principles. It has since been reengineered to include all the requirements of an HACCP program.

The acceptance of greater responsibility by industry in monitoring and controlling their processes in compliance with the regulations has permitted FSPD to assume an auditing role and focus on areas where there is higher risk. The resulting efficiencies in the systems have allowed FSPD to continue to deliver a comprehensive inspection program that provides even greater assurances that Canadian fish products meet international market requirements. In many cases, QMP allowed industry to take on monitoring and controlling critical health risks that were once the responsibility of government. This was only possible because the QMP provided a structure for nongovernment inspectors to assess health and safety concerns that could be audited by regulators.

Another example is monitoring, control, and surveillance. Monitoring, control, and surveillance (MCS) refers to the systems and processes used to gather complete and accurate information about a fishery and ensuring that a fishery is conducted in accordance with a fisheries management plan and fisheries regulations to control the fishing practices of industry. Dockside monitoring is the process used in most fisheries, except the lobster fishery, to gather information about landings by individual fishers. Fishers procure the services of a dockside monitoring organization to monitor their landings of fish and report them to the Department. The Auditor General of Canada found several instances of deficiencies with the program when it audited in 1997 (Canada, 1997):

- The Department had not quantified the potential error resulting from misidentification of the species and weigh of incoming fish;
- The dockside monitoring program is not always carried out by organizations that are at arm's length with the harvesting sectors in the region;
- The dockside observers would not be able to ensure that all fish landed would be included in the Department's catch data systems.

LIABILITIES

Recently, in the province of Ontario, Canada, water contaminated by *Escherichia coli* O157 caused 2,000 illnesses in the small town of Walkerton. The majority of reported cases occurred after 12 May 2000 and continued until late June 2000. Of the 1,346 reported cases identified, 1,304 were considered to be primary or caused by direct exposure to Walkerton municipal water. The estimated overall number of cases associated with the outbreak exceeded 2,000. Sixty-five patients were admitted to a hospital, and of these, 27 developed hemolytic uremic syndrome. Six people died as a result of the outbreak (Canada, 2000).

A series of unfortunate circumstances occurred to cause an outbreak of this magnitude. These included heavy rains accompanied by flooding, *E. coli* O157:H7 and *Campylobacter spp.* present in the environment, a well subject to surface water contamination, and a water treatment system that may have been overwhelmed by increased turbidity. Bacterial monitoring can only identify a contaminated source after the contamination has spread through the water system and put the public at risk. However, compounding these issues were policy decisions made by the Ontario government.

In 1996, Eva Ligeti, the environment commissioner at the time, admonished the government for compromising environmental protection, pointing specifically at the testing of drinking water. For

years, the Ministry of Environment and Energy and the Ministry of Health provided drinking water testing to municipalities. With little notice, these services were transferred to municipalities that had to find testing labs and the money to pay for them. The public was not consulted, nor were municipalities (National Post, 2000). The Ontario Ministry of the Environment operating budget was cut 42.4 per cent from $287 million in 1994/1995 to $165 million for 2000/2001.

CONCLUSIONS AND RECOMMENDATIONS

To address these liabilities, a sampling program must be carefully designed, implemented, audited, and documented. Some of the parameters of the program that must be considered are the activities (patrol, water or biotoxin sampling), responsibilities of various personnel, the control of records, the standard operating procedures for sampling, the control and calibration of sampling equipment, and the conditions for shipping to laboratories. Additional control must be implemented if private laboratories are being utilized. Finally, a plan must be in place for the control of nonconformities in sampling.

Government must directly audit these programs. They must seek their own data to compare with that collected by the nongovernment authority. Within the relationship with these samplers there must be a direct authority to control their activities. This audit and control program must be documented so as to allow audits by other government departments or foreign governments. An important component of the audit is the assessment of the efficiency and efficacy of the program.

The nongovernment authorities that carry out these activities and the observers whom they employ must be independent of the fishers they monitor. The effectiveness of their program must be audited by the nongovernment controlling authority, and corrections and amendments to the program must be made. The human resource requirements of the sampling programs must be adequately addressed by the needs of personnel, resources, and training of these people. Training must be conducted according to a standardized curriculum developed by the government body. In some cases, this can be problematic because training of government employees has, in some areas, ranged from formal classroom to new staff being presented with keys and a copy of the regulations. If carefully planned and implemented, a program utilizing nongovernment samplers can be a useful addition to a government's shellfish sanitation program.

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DEPURATION OF *ESCHERICHIA COLI* FROM SYDNEY ROCK OYSTERS AFTER HARVESTING AND STORAGE. **Iona Reid, Paul Heiskanen, Graham H. Fleet,** and **Ken A. Buckle,** Department of Food Science and Technology, The University of New South Wales, Sydney, NSW 2052, Australia

In New South Wales, Australia, it is mandatory to depurate oysters for 36 h at ambient temperatures (14–25°C) prior to sale. As a consequence of commercial and marketing demands, it is common practice for farmers to harvest and store oysters out of water for periods up to 7 days prior to depuration. It is not known if this practice affects the efficiency of depuration with respect to elimination of microbiological pathogens. In Australia, oyster safety is measured using the indicator organism *Escherichia coli*, where the legislative limit at which oysters can be sold after depuration is 2.3 *E. coli*/g of oyster flesh.

To determine if storage affects depuration efficiency, experiments were designed in which freshly harvested Sydney Rock oysters were artificially contaminated with *E. coli* to levels of 10^2–10^3, stored for 0, 4, 7, and 10 days at 15°C and 23°C, and then tested for their ability to eliminate *E. coli* during depuration. Depuration conditions (salinity 35 ppt, water temperature 14°C and 23°C) were chosen to reflect the estuary conditions from where the oysters were harvested. Each storage experiment was conducted in triplicate. During depuration, samples consisting of 10 oysters each were removed at 0, 6, 12, 24, 36, and 48 h. Oysters were analyzed for *E. coli* using the direct plating method of Anderson and Baird-Parker. Samples were plated onto Tryptone Bile Agar plates overlaid with nitrocellulose membranes and tested using indole reagent as detailed in the Australian Standard (AS 1766).

During storage of contaminated oysters at both 15°C and 23°C, it was noted that *E. coli* levels within the oyster prior to depuration decreased by 95% from day 0 to day 10. This has important implications for the reliability of using *E. coli* as an indictor of safety in oysters.

Oysters stored for up to 10 days met the legislative standard of 2.3 *E. coli*/g after depuration for 36 h. However, stored oysters exhibited some mortality during depuration. After 7 days of storage, mortality rates during depuration and after storage at 15°C and 23°C were less than 10%. After 10 days storage at 15°C, 24% mortality was observed during depuration, whereas at 23°C only 3% mortality was observed. The death and loss of oysters has economic implications to the farmer. The Code of Practice for Depuration of Oysters in New South Wales (Anon, 1999) specifies that Sydney Rock oysters must not be stored for more than 4 days prior to use in depuration. Our data suggest that it may be possible to extend this requirement to 7 days, thereby providing oyster farmers with more flexibility in their production and marketing schedule, without compromising safety as measured by the *E. coli* standard.

EFFECT OF SALINITY AND TEMPERATURE ON DEPURATION EFFICIENCY OF THE SYDNEY ROCK OYSTER (*SACCOSTREA COMMERCIALIS*). **Paul Heiskanen, Iona Reid, Graham H. Fleet,** and **Ken A. Buckle,** Department of Food Science and Technology, The University of New South Wales, Sydney, NSW 2052, Australia

Sydney Rock oysters (*Saccostrea commercialis*) are cultivated in estuaries along the New South Wales coastline, which extends over a distance of 1,400 km from north to south of the state. Depuration is mandatory for all oysters offered for sale in New South Wales and is governed by a Code of Practice for Depuration of Oysters in New South Wales (Anon, 1999), which prescribes parameters such as water temperature (between 14°C and 25°C) and salinity (greater than 18 ppt). After depuration, oysters must conform to the bacteriological standard, namely less than 2.3 *Escherichia coli*/g. Water temperatures in harvest areas may range from 10–28°C, and salinities from fresh (0 ppt) to 35 ppt, depending on geographical location, season, and climatic conditions. Hence the permitted depuration conditions place a commercial constraint on farmers because there may be a requirement to heat or cool the water or increase water salinity, depending on the location and the season. Commercial farmers consider that oysters adapted to their growing conditions should depurate effectively at these conditions to reach the required level of less than 2.3 *E. coli*/g in 36 h, as stipulated in the regulations. Consequently, they believe it should not be necessary to adjust and monitor depuration water to meet the temperature and salinity limits of the Code of Practice.

Experiments were designed to examine the efficacy of depuration of Sydney Rock oysters under extremes of salinity and temperature for particular estuaries. Oysters were artificially contaminated with *E. coli* to 10^2–10^3 cfu/g under conditions that simulated growing conditions, as it was not always possible to obtain naturally contaminated oysters. The oysters were then depurated for 48 h in pilot scale plants in the laboratory under controlled salinity and temperature. Depuration efficiency was measured by the elimination of *E. coli* from oysters using the method of Anderson and Baird-Parker. Samples taken after 0, 6, 12, 24, 36, and 48 h were plated onto Tryptone Bile Agar overlaid with nitrocellulose membranes and tested for indole production to indicate *E. coli* as described in the Australian Standard (AS 1766).

To date, oysters cultivated from two geographical locations have been examined. The first estuary (Hawkesbury River, Central Coast, north of Sydney) experiences temperature and salinity ranges of 14–25°C and 15–35 ppt, respectively. Experiments were carried out on all combinations of 14°, 19°, and 25°C and 15, 25, and 35 ppt, giving a total of nine sets of depuration conditions. Depuration was successful at all combinations of temperature and salinity except for those at 15 ppt, where spawning was induced. The combination of low salinity and higher temperatures also accelerated the onset of spawning. The second estuary (Pambula Lake, south of Sydney) experiences temperature ranges of 10–

22 C with relatively stable salinity (32–35 ppt). Experiments were carried out at 35 ppt salinity and water temperatures of 10°, 12°, 14°, 16°, 18°, and 20°C, with these parameters closely matching growing conditions at the time. Depuration was consistently effective at 14°, 16°, 18°, and 20 C, but at 10° and 12°C some oysters failed to meet the *E. coli* standard.

Further work on estuaries at other locations is currently underway. At the completion of the study, alterations to the Code of Practice will be recommended to accommodate acclimatization of oysters to a wider range of temperatures and salinities, thereby balancing the practicality and economics of commercial processing without compromising end product safety.

NONSELECTIVE ACCUMULATION OF PSP TOXINS IN MUSSELS *MYTILUS GALLOPROVINCIALIS* FED WITH TOXIC DINOFLAGELLATE *ALEXANDRIUM TAMARENSE*. **Toshiyuki Suzuki, Kazuhiko Ichimi,** and **Makoto Yamasaki,** Tohoku National Fisheries Research Institute, 3-27-5 Shinhama, Shiogama, Miyagi 985-0001, Japan. E-mail: tsuzuki@affrc.go.jp

Comparison of paralytic shellfish poisoning (PSP) toxin profiles between natural mussels *Mytilus galloprovincialis* and toxic dinoflagellate *Alexandrium tamarense* collected at the same monitoring site of Ishinomaki Bay, Japan, was carried out by high-performance liquid chromatography (HPLC). The prominent toxins in the mussels and *A. tamarense* were *N*-sulfocarbamoyl toxins (C1 and C2) and carbamate toxins (GTX1 and GTX4). The proportion of carbamate toxins (GTX2 and GTX3) in the mussels was significantly higher than that of *Alexandrium tamarense*.

Mussels *M. galloprovincialis* were reared with the toxic dinoflagellate *A. tamarense*. Changes in both PSP toxin contents and the toxin profiles of mussels during the feeding experiment were monitored by HPLC, and the toxin profiles of mussels were compared with that of *A. tamarense* fed by the mussels. The prominent toxins in the mussels and *A. tamarense* were *N*-sulfocarbamoyl toxins (C1 and C2) and carbamate toxins (GTX1 and GTX4). The toxin profiles of both mussels and *A. tamarense* were almost constant during the feeding experiment. In contrast to the result obtained in the field experiment, remarkable differences in the toxin proportion between mussels and *A. tamarense* were not observed.

These results indicate that the increase of the proportion of carbamate toxins (GTX2 and GTX3) in mussels took place after the accumulation of toxins and denies the selective accumulation of toxins in mussels.

IDENTIFICATION OF *E. COLI* SOURCES FOR THE PECONIC ESTUARY WATERSHED FOR EFFECTIVE MITIGATION OF NONPOINT SOURCE POLLUTION. **Emerson Hasbrouck,[1] Lori Racaniello,[1] George Simmons,[2]** and **Sue Herbein,[2]** [1]Cornell Cooperative Extension Marine Program, 3059 Sound Avenue, Riverhead, New York 11901; and [2]Virginia Polytechnic Institute and State University, 2119 Derring Hall, Blacksburg, Virginia 24061

Studies such as the N.U.R.P. Study and the L.I. 208 Study have identified nonpoint source pollution as a major factor affecting water quality on Long Island. The BTCAMP Study and the Peconic Estuary Program Action Plan identify nonpoint source pollution as a significant contributor to surface water quality problems in the Peconic Estuary.

One of the major components of stormwater runoff is coliform bacteria. Coliforms are an indicator of the possible presence of pathogenic organisms and are used by various agencies to determine water quality. Often the most puzzling aspect of nonpoint source mitigation is determining the exact source of pollutants and hence the best means of remediation. Failing septic systems and wildlife are often credited as major contributors of bacteria to stormwater runoff. However, they require different remediation strategies. In fact, different animal groups also require different remediation strategies.

As a means to identify coliform sources, a DNA library, specific to Eastern Long Island, is being developed based on *Escherichia coli* isolated from the scat of animals (including humans) that live in association with estuaries of Long Island. The DNA library consists of "genetic fingerprints" from pulsed field gel electrophoresis for each *E. coli* isolate. The next step in the process is to take water samples from impacted areas, isolate out pure strains of *E. coli*, and then compare their genetic profile to the known sources in the DNA library. Matches with known sources will identify the animals or animal groups contributing coliform bacteria to the selected water body.

This project will expand the identification process and will additionally contribute to expanding the DNA library. Once the input source or sources have been identified, appropriately tailored remediation strategies can then be developed. Results will provide the ability to identify the animal sources of *E. coli* in nonpoint source pollution and in impacted embayments to develop unique and innovative tools to assist managers in developing and implementing the most appropriate and cost-effective management approaches to stormwater discharge and other nonpoint source pollution remediation efforts.

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COVER PHOTO: A European green crab, *Carcinus maenas*, caught in Coos Bay, Oregon in the summer of 2001. This 94 mm male most likely settled from the plankton early in 1998. (Sylvia Yamada and Tim Davidson)

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